Supporting Information

A Sonosensitizer-based Polymeric Nanoplatform for Realizing Chemo-sonodynamic Combination Therapy of Lung Cancer

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**Experimental**

**Materials and devices**

CS, Mw: 10 kDa, Xuzhou Jiangda Biotechnology Co., Ltd. (Xuzhou, China) and DTX, Heowns Biochemical Technology Co. (Tianjin, China) were employed in acceptance status. ADH (>99.0%), 1-ethyl-3-(3-dimethylaminopropyl)-cabodiimide hydrochloride (98 %, EDC), and N-hyodroxy succinimide (NHS) were both purchased and obtained from Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). Rh (> 98 %) was acquired from MREDA Technologies. Co., Ltd. (Beijing, China). DTT (99 %) was obtained from Solarbio Science & Technology Co., Ltd (Beijing, China). LA (98%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Coumarin 6 (C6, 98 %), [Cell Counting Kit8](https://b2b.baidu.com/b2bsearch/jump?url=https://www.912688.com/supply/323066515.html&query=CCK-8&logid=4050804576&srcId=27729&category=%E5%8C%96%E5%B7%A5%E8%83%BD%E6%BA%90;%E5%8C%96%E5%AD%A6%E8%AF%95%E5%89%82;%E5%85%B6%E4%BB%96%E5%8C%96%E5%AD%A6%E8%AF%95%E5%89%82&sv_cr=0&uign=5a6851d1985c0fe126ab3406959afd67&iid=59288da58888137f1455827c57de5d4d&timeSignOri=1577775269&xzhid=28406439&miniId=8469&ii_pos=0&from=b2b_straight&srcid=5103&from_restype=product) (CCK8), and N-acetylcysteine (NAC) were attained from Sigma-Aldrich (USA) for different *in vitro* usage. Fetal bovine serum (FBS), DMEM as well as RPMI-1640 medium were received and paid from Gibco BRL (Gaithersberg, MD, USA). All other materials and reagents in this article were selected in the level of analytical grade.

Therapeutic ultrasound devices: Medical ultrasonic therapy instrument (DM-300B, purchased from Shenzhen Dimip Technology Co., Ltd, China) was aimed at realizing ultrasound stimulation (1MHz), duty cycle (10%-90%, 40% was applied) and pulse repetition cycle (10 ms). The acoustic intensity could vary in the range of 0.3 to 3.0 W/cm2. Considering the clinical practice and safety, the acoustic intensity of 1.2 W/cm2 was applied. Meanwhile, the all [parameters](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) maintained consistency during the whole experiment for SDT studies.

Adenocarcinoma human alveolar basal epithelial cells (A549) together with the other human NSCLC cells (H1299) were kindly obtained from the Institute of Biochemical & Biotechnological Drug, Shandong University. However, these two different cells were grown in RPMI 1640 and the DMEM complete medium, respectively, and mixed with 10% FBS, 250 µg/ml amphotericin B, 100 U/mL penicillin G, as well as the 100 mg/mL streptomycin. Subsequently, the culture plates were placed in a thermostatic incubator at 37 C°, 5% CO2.

Balb/c nude mice (6-8 weeks) were acquired and properly fed from Beijing Vital River Laboratory Animal Technology Co. Ltd. All animal studies were conducted after the approval of Institutional Animal Care and Use Guides.

**Polymer Synthesis**

The final synthesis of CS-ADH-Rh-LA material was conducted by triple steps of amide reaction (Fig. S1). First, the amine terminals of ADH were coupled with carboxylic groups of CS to synthesize CS-ADH with some modifications [[1](#_ENREF_1)]. Then, 2.0 g EDC and 0.8 g NHS were added to the pre-dissolved CS in distilled water (DW, with the concentration of 10 mg/mL), 15 min before adding 3.52 g ADH. The reaction by stirring was conducted for 24 h at 25℃. Finally, the product was dialyzed (MWCO 3500) against the DW to remove the residual agents, following lyophilization. The assaying of ADH was quantified by comparing the integration proportion in 1H-NMR spectra of ADH and CS.

Rh was grafted to the CS-ADH through the same method for synthesis of Rh-lysinate. The Rh-CS-ADH was successively synthesized by trial and error method because of lack of the available method[[2](#_ENREF_2)]. Firstly, EDC and NHS (5 equiv. / Rh, each) were added into Rh in DMSO for 4h under a nitrogen atmosphere in dark. The next task is to dissolve 100mg CS-ADH in a volume of 10 mL mixed solvent (DW: DMSO (1:1)), then subsequently added to the Rh solution (the proportion of Rh to CS-ADH varied from 1:10 to 3:10). The reaction was continued for 24h away from light at 25℃, following by dialysis (MWCO 3500) against DMSO and DW, and lyophilization. The degree of substitution (DS) of Rh was detected by the instrument of UV-Visible Spectrophotometer (UV-Vis) at 443 nm.

LA was jointed to CS-Rh conjugates through the reaction of [amidation](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) coupling of LA with ADH [[1](#_ENREF_1)]. To activate carboxylic groups, LA was first reacted with similar amounts (2 equiv. / LA) of EDC as well as the NHS for 12 h in dimethyl formamide (DMF). Next, CS-ADH-Rh (0.25 mmol) was put into the DW and DMF (1:1) mixture of 10 mL, then LA-NHS ester (0.35 mmol) was added. After continuing for 12 h at 25℃ without light, the end acquisition was purified against DMF (MWCO 3500) following DW, before being lyophilized. Finally, the CS-ADH-Rh-LA was constructed, and the DS of LA was quantified by UV-Visible spectrophotometer at 335 nm. Further, the CS-Rh-LA polymer with different ratio of LA to CS-Rh (0.1: 1 to 0.3:1) was synthesized, followed by detecting their critical micelle concentration (CMC).

The NMR and the Fourier transform infrared (FT-IR) methods were both used for determination of successful conjugation. To record 1H-NMR spectra, CS or CS-ADH were respectively dissolved in D2O, while for 13C-NMR spectra, CS-ADH-Rh or CS-ADH-Rh-LA copolymers should be fully dissolved in mixed solution of DMSO-d6/D2O (1:1, v/v).

**Preparation and characterization**

The sonication method was applied to construct the non-crosslinked CS-Rh-LA nanoparticles (NC-NPs). Briefly, the synthetic polymer of CS-ADH-Rh-LA conjugates were added and completely dissolved in DW by stirring, and sonicated for 12 min by the ultrasonic probe (120W, 4s off / 2s on), and later on it was purified by 0.8 μm membrane filter. For the construction of cross-linked nanoparticles, DTT (10% content of easter group in NC-NPs) solution was added in the NC-NPs after sonication, and stirred for 12 h. Thereafter, NC-NPs mixture was dialyzed against DW followed by filtration and lyophilization. Therefore, the cross-linked nanoparticles (C-NPs) were acquired and stored under 4℃.

With regard to C-NPs encapsulating DTX, the previous prepared copolymer was mixed with DTX methanol solution (100 μL) dropwise to CS-Rh-LA in DW (1 mL), stirring for 5 h at 25℃. Later on, this resulting solution was sonicated for 12 min and dialyzed (MWCO 3500) to remove methanol. The cross-linked NPs encapsulating DTX (DTX/C-NPs) were prepared following the previous method. Therewith it was placed in the centrifuge at 4000rpm for 15min, and later on the supernatant was push through 0.8 μm membrane filter. It was worth noting that all the operation should be made in the dark.

The content of DTX was determined by high performance liquid chromatography (HPLC) which was subsequently equipped with Hypersil-BDS C18 column (5 μm, 250 mm×4.6 mm) and detection wavelength of 227 nm. Mobile phase was composed of the mixture of H2O and CH3CN (45:55 (v/v)), which flowed at a constant speed of 1.0 mL/min. In addition, the values of drug loading (DL) as well as the entrapment efficiency (EE) were calculated according to these equations:



The *in vivo* stability of both C-NPs and NC-NPs was evaluated in the medium of PBS and 0.1M NaCl, and the dilution stability against 1000-fold water was also evaluated. For C-NPs, the stability in 10% FBS was also evaluated. The NPs were cultured at 37 C° and 100 rpm, and size was tested by DLS at 25 C°. Besides, the morphology of C-NPs and NC-NPs was photographed via transmission electron microscopy (TEM, Japan).

**Critical micelle concentration (CMC)**

Employing method of [fluorescence](C:/Users/dell/AppData/Local/youdao/dict/Application/8.9.3.0/resultui/html/index.html#/javascript:;) [detection](C:/Users/dell/AppData/Local/youdao/dict/Application/8.9.3.0/resultui/html/index.html#/javascript:;), the CMC was detected by the substance of pyrene probe [[3](#_ENREF_3)]. Briefly, the pyrene was dissolved in acetone to make the final concentration of 2.5×10-4 M, and the NC-NPs solutions with the concentration range from 1 ng/mL to 1 ug/mL were added to the pyrene after the acetone was evaporated. The [fluorospectro](C:/Users/dell/AppData/Local/youdao/dict/Application/8.9.3.0/resultui/html/index.html#/javascript:;) [photometer](C:/Users/dell/AppData/Local/youdao/dict/Application/8.9.3.0/resultui/html/index.html#/javascript:;) (Hitachi, Japan) was applied for recordation of emission spectra (Ex:334nm). Results was obtained by drawing the plot of I373/I384 against its logarithmic concentrations.

**De-crosslinking and drug release of C-NPs triggered by DTT [**[**4**](#_ENREF_4)**]**

The C-NPs or NC-NPs were respectively dissolved and cultured with 20 μM and 20 mM DTT in [sodium](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) [acetate](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) solution at 37 C° for 24h, and DLS was applied to determine the size distributions at different time points. Besides, the morphology of NPs was observed by TEM before or after adding the DTT solution.

The *in vitro* drug release behavior was investigated in PBS (7.4, 0.1M), PBS containing 20 μM and PBS containing 20mM DTT solutions. Briefly, the DTX-loaded C-NPs were cultured within dialysis bags against 35 mL solution in a shaking bed (37℃, 100 rpm) for 0.5 h to 72 h. At corresponding time, 1 mL of different media containing DTX was fetched for detection by HPLC, while [isopyknic](C:/Users/dell/AppData/Local/youdao/dict/Application/8.9.3.0/resultui/html/index.html#/javascript:;) blank medium was replenished subsequently. Each sample was carried out in triplicate.

**Hemolysis Test**

Blood was collected from rabbits, stored in heparinized-tubes and put into a centrifugal (3000rpm, 10min) to separate red blood cells. The pellet as rinsed with saline for three times after the supernatant was discarded, following centrifugation. In the end, red blood cells were re-suspended with a final content of 2% in saline. Next, C-NPs solutions with different concentrations (0.1, 0.25, 0.5, 0.75, 1.0 mg/mL) were instilled into above obtained erythrocytes solution. Soon after, the mixed C-NPs solution was cultured for 1h at 37C°, followed by centrifugation. Finally, the supernatants were collected and scanned with UV-Visible spectrophotometer at 541 nm. The study was carried out in triplicate.

**Intracellular uptake**

In order to study the intracellular uptake of nanoparticles, the fluorescent model drug, C6, was encapsulated into the C-NPs (C6/C-NPs). A549 cells were inoculated on 12-well plate at 1.2×104 cells/well. After 75% confluence, the original media were discarded and changed to three different media of 2 μg/mL C6 (free drug group), nanoparticles encapsulating 2 μg/mL C6 (C-NPs group), and normal medium (control group), respectively. At different intervals, the uptake was terminated by washing cells with PBS thoroughly. Then cells were collected in different tubes for detection in flow cytometer (FCM).

To track the internalization mechanism of C-NPs, the confocal laser scanning microscopy (CLSM, Zeiss, Switzerland) was applied. The A549 and H1299 cells were incubated in 12-well plate with the density of 8×103 cells/well, and fresh media listed above were added until 75% confluence was achieved. Additionally, cells were cultured with free CS (10 mg/mL) 1h before adding the C-NPs solution, constructing the CD44 receptors inhibiting group. After the incubation, Hoechst 33342 was applied to mark the nuclei. Therewith, CLSM result was observed after washing with PBS for 3 times.

**Transcellular delivery**

The transcellular delivery of C-NPs was investigated with A549 cells. In brief, the cells were plated and cultivated respectively on coverslips (I)-(III) (Fig. 4B) with the density of 1×104 cells/well overnight [[5](#_ENREF_5)]. In the first place, cells on coverslip (I) were incubated with C6-loaded C-NPs (2 μg/mL of C6) for 4h. Then coverslip (I) was washed with PBS for three times, shifted to the fresh medium, and cultured with coverslip (II) for 12h. Identical to above steps, coverslip (II) was washed and co-cultured with the coverslip (III) for further 12h. In the end, all the coverslips rinsed by PBS following staining by Hoechst 33342 (10min) were observed with CLSM.

**In vitro cytotoxicity**

Rh, CS-ADH-LA, C-NPs and DTX/C-NPs were measured for their cytotoxicity through CCK-8 kit using A549 and H1299 cells. In the first place, cells were cultivated on a 96-well plate with the density of 5×103 cells/well, containing 100μL complete medium. Then, C-NPs containing 0, 0.1, 0.5, 1, 2, 3, 4, 8, 16, 32 μg/mL of Rh were added and cultivated for 24h. Cells were washed twice and changed fresh media. To investigate the dark toxicity, the plates were placed in dark for further 24h before adding CCK-8 kit. While for SDT group, the cells should be suffered from ultrasound with 1.2 W/cm2 for 1, 3, as well as 5 min, and CCK-8 assay was performed after 24 h. For synergistic cytotoxicity evaluation, cells were incubated with DTX/C-NPs for 24 h (Rh from 0.001 to 10 μg/mL, while DTX ranged from 0.012-120 ng/mL). After rinsed by PBS, cells were also dealt with/without SDT and cultured according to the previous step. The results were recorded by [microplate](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) [reader](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) at 450nm, while equation 3 below was applied to calculate cell viability.



The value of ODs represented the absorbance of samples, while the signal of ODb and ODc represented the absorbance of blank and control groups, respectively.

**In vitro apoptosis study**

The apoptosis detection kit (Solarbio) was applied to study the *in vitro* apoptosis. A549 cells were exposed to blank medium, free Rh (2.5 μg/mL), CS-ADH-LA (132 μg/mL), C-NP (containing 2.5 μg/mL Rh), and DTX/C-NPs (containing 2.5 as well as 14 μg/mL of Rh and DTX) for 12 h, respectively. After being washed with PBS and replenishing new medium, cells in SDT groups were all suffered from persistent ultrasonic therapy for 3 min. After 4h, cells were stained and analyzed for apoptosis and necrosis using FCM.

**Detection of** **live/dead cells**

To observe the distribution of live/dead cells, A549 cells were plated on 14 mm petri-dishes (8×103 cells/well) and then cultured nearly for 12h. After treatments with blank medium, free Rh (2.5 μg/mL), CS-ADH-LA (132 μg/mL) and DTX/C-NPs (2.5 μg/mL of Rh, 14 μg/mL of DTX) for 12h, respectively, old medium was discarded and cells were washed and cultured with fresh media in an incubator for 4 h. Then Calcein-AM/PI Kit with the aim at staining different was applied following manufacturer’s instruction. While, final results were recorded by CLSM.

**ROS detection**

Generation as well as variation of ROS in cancer cells were measured *in vitro* by using ROS detection kit (Beyotime Biotech, China). A549 cells were seeded in 12-well plates/14mm size petri-dishes at 1.0×104 / 8×103 cells/well. After 24 h, old medium was replaced with different culture solutions of fresh media, free Rh (2.5 μg/mL) and C-NP (containing 2.5 μg/mL Rh). After continuing cultivation for 4 h, 10 μM DCFH-DA probe diluted with fresh media was added to the cells, and the SDT groups were treated with ultrasound (1.2 W/cm2, 3 min). After culturing in the [cell](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) [incubator](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) for 20-30 min, cells were washed, collected, followed by analyzation using FCM to acquire the fluorescence intensity. While for the CLSM observation, the treated cells in petri-dishes were stained with Hoechst 33342 to mark the cell nuclei, and observed after being washed with PBS. For inhibitory studies, the ROS scavenger NAC of 5 mM concentration was pre-added to the culture media 1 h before treating with C-NPs, and the samples were observed by CLSM with other groups.

For further study, the cells were plated on 14 mm petri-dishes (8×103 cells/well) followed by incubating overnight. Firstly, cells were cultivated by C-NPs at different concentrations of Rh (1, 2.5 and 5μg/mL of Rh) for 12 h, and the DCFH-DA probe was loaded as mentioned above. Subsequently, the cells were exposed to ultrasound and sequentially cultured for 4 h, and cell nuclei were stained to observe under CLSM. Secondly, the cells were incubated with C-NPs (5μg/mL of Rh) for different time points (2, 4, 8 h), and treated as mentioned previously.

**Singlet oxygen**

Fluorescence probe 9,10-dimethylanthracene (DMA) was used to detect singlet oxygen. Briefly, DMA was dissolved in DMF and added to different solutions of ethanol, Ce6 (5 μg/mL in ethanol), Rh (5 μg/mL in ethanol), C-NPs (containing 5 μg/mL Rh in ethanol), and EMO (5 μg/mL in ethanol), making the final concentrations of DMA to 20 μM. After treatment with ultrasound (1.2 W/cm2, 3min) followed by continued incubation for 6h, solutions were scanned with [fluorescence](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) [spectrophotometer](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) (Ex = 360 nm, Em = 380-550 nm). The spectrum was recorded for the comparison of the fluorescence intensity.

**Mitochondrial membrane potential (MMP) detection**

The MMP detection kit (JC-1, Beyotime Biotech, China) was applied. Briefly, cells plated on 6-well plates (1.2×10/4 cells/well) for nearly 75% confluence were cultivated by free medium and DTX/C-NPs (containing 2.5 and 14 μg/mL Rh and DTX) for 12 h. After rinsing by PBS, cells were treated by the ultrasound (1.2 W/cm2) for 1, 3 and 5min, respectively. 12 h later, cells were harvested and washed twice followed by centrifugation, and the jc-1 fluorescence probe was added. After cultivation for 30 min at 37 ℃ without light, cells were washed and harvested for the detection by FCM.

**Cell cycle analysis**

Cell cycle was also evaluated through FCM using cell cycle detection kit (Keygen Biotech, China) [[6](#_ENREF_6)]. In the first place, cells were seeded in 6-well plate (1.2×10/4 cells/well) followed by cultivating overnight. Then cells were incubated with the fresh medium, C-NPs (2.5 μg/mL of Rh), DTX (containing DTX of 14 μg/mL) and DTX/C-NPs (2.5 and 14 μg/mL of Rh and DTX) for 12 h. After being washed and replaced with fresh media, cells were sonicated for 3 min following by culturing for 12h at 37°C. Later on, cells in different solutions were harvested and stained with the diluted PI/RNase A solution in the dark for 60 min, and were measured by FCM. The results were analyzed by the ModFit LT software to acquire the corresponding DNA histograms.

**Cellular microtubule regrowth assay**

The microtubule structure was measured by Tubulin-Tracker Red (Beyotime Biotech, China) fluorescence probe and CLSM [[7](#_ENREF_7)]. Briefly, cells were plated and cultured for 12h on 14 mm petri-dishes (8×103 cells/well). Later on, old medium was replaced with fresh medium, C-NPs (2.5 μg/mL of Rh), DTX (containing DTX of14 μg/mL) and DTX/C-NPs (2.5 and 14 Rh and DTX), respectively, then co-cultured for 4h at 37°C. In the following step, cells were rinsed and fixed with paraformaldehyde for 10 min, then stained with the kit. Finally, Hoechst 33342 was applied for staining of cell nuclei, and the microtubules were acquired.

**Subcellular localization of nanoparticles**

For the sake of observing the subcellular localization, C-NPs were labeled with C6 by encapsulating it into the hydrophobic core as mentioned previously. A549 cells were first seeded in the petri-dishes (8×103 cells/well) for 12 h, then incubated with C6/C-NPs (2 μg/mL of C6) for 1, 3, 6, 12 h, respectively, and rinsed with PBS. Following, the cells were fixed and stained by 50 nM Lyso-Track Red (Beyotime Biotech, China) for 1 h, 20 nM Mito-Tracker Red CMXRos (Beyotime Biotech, China) for 0.5 h, and 166.5 μg/mL Golgi-Tracker Red (Beyotime Biotech, China) for 0.5 h, respectively, to monitor the C-NPs distribution in lysosome, mitochondria and Golgi apparatus. Hoechst 33342 were used to mark the cell nuclei before the observation using CLSM.

**Immunofluorescence assay**

A549 cells were seeded on the sterile microscope cover glass (3×103 cells/well), incubated overnight, and replenished with fresh media, C-NPs (2.5 μg/mL of Rh) as well as DTX/C-NPs (containing Rh and DTX for 2.5 and 14 μg/mL), respectively, for further cultivation of 121h. Then, cells were rinsed with PBS, and some SDT groups were treated with ultrasound (1.2 W/cm2, 3 min). After 12 h, cells were fixed as well as permeabilizing using 0.2% Triton X-100. After blocking for 30 min, cells were cultured with GM-130 antibody (DF7556, Affinity Biosciences, USA) overnight in bovine serum albumin (1%, BSA) that was dissolved by PBS at 4℃. After washing with 1% BSA before adding the secondary antibody of goat anti-rabbit IgG (H+L) Fluor 594-conjugated-S0006 (Affinity Biosciences, USA). Later, the cells were stained using Hoechst 33342 to acquired intact images by CLSM.

**Cell migration and invasion**

The nanoparticles were evaluated for cell migration and invasion as previously described [[8](#_ENREF_8)]. For the evaluation of cell migration and repair capability, A549 cells were plated on 12-well plates and cultivated to achieve full confluence. Sterile 200-μL pipet tips were applied to cell monolayer by scratching in straight lines, forming a clear zone without cells. After being washed and replenished with 2 mL fresh medium or media including free Rh (0.5 μg/mL), C-NPs (0.5 μg/mL of Rh), DTX/C-NPs (0.01 as well as 0.056 μg/mL of Rh and DTX) and DTX (0.1 μg/mL of DTX), respectively. After incubating for 24 h, the SDT groups were treated with ultrasound (1.2W/cm2) for 3 min, and hatched for further 4 h. Images were acquired at 0 and 28 h after treatment, and the wound healing rate was calculated.

For the evaluation of transwell invasion study, A549 cells were incubated for 12 h with different solutions of fresh medium, C-NPs (0.5 μg/mL of Rh) as well as DTX/C-NPs (0.01 or 0.056 μg/mL of Rh and DTX), and SDT groups were treated with ultrasound (1.2 W/cm2, 3min). Thereafter, cells were collected and dispersed among the FBS free media (5×104 cells/well) and placed on the upper chambers, which was coated with diluted caky Matrigel (M8370, Solarbio, China). While in the lower chamber, the complete culture media with 10% FBS acted as the chemokine. 24 h later at 37 ℃, the wells were rinsed before fixing with 4% paraformaldehyde. Then the upper Matrigel was wiped off using the [cotton](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) [swab](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;), thereafterthe crystal violet staining solution (Beyotime Biotech, China) was applied to cell staining for 15min. And images were acquired by an inverted microscope (NIKON Ti-U, Japan).

**Western Blot assay for SDT induced apoptosis pathway**

A549 cells were seeded in the 10 cm petri dishes (3×106 cells/well), then incubated for 12 h before treating with free medium or different formulations containing C-NPs (2.5 μg/mL of Rh) as well as DTX/C-NPs (2.5 as well as 14 μg/mL of Rh and DTX). Then cells were rinsed by PBS and replenished with 10 mL free media, and the SDT groups were treated with ultrasound (1.2 W/cm2,3min). Next, all the groups were cultured for 12 h before harvesting and lysing the cells in RIPA buffer that was dissolved in 1% phenylmethanesulfonyl fluoride (PMSF). The variation of apoptosis-related proteins such as MMP9, VEGFA, Caspase-3, as well ascleaved-Caspase-3 was determined, mainly through sulfate-polyacrylamide gel electrophoresis gel (SDS-PAGE) followed by transferring onto the polyvinylidene fluoride membranes. The membrane was immunoblotted by primary antibodies mentioned above as well as β-actin for the [internal](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) [reference](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;)s, respectively. Subsequently, the horseradish peroxidase-labeled second antibodies were co-incubated with the samples aiming at tagging the primary antibodies. In the end, the protein bands were detected with the help of enhanced chemiluminescence reagents.

**Construction of tumor-bearing mice model**

To investigate the tumor inhibition effects, the unilateral or bilateral tumor models were constructed for *in vivo* imaging or tumor suppression assays, respectively. Firstly, A549 cells with density of 1×107 cells in 100 μL were injected into right flanks, and the same amount of A549 cells were injected into left flanks four days after that first injection to construct the bilateral models. After culturing for nearly 3 weeks, mice were divided into 6 groups at random for treating with NS, C-NPs + DTX, Taxotere®, DTX + Rh, DTX/C-NPs, and DTX/C-NPs + SDT, respectively.

**In vivo biodistribution**

The aim of bioluminescent imaging system (IVIS Kinetic, USA) was to capture the imformation in tumor about C-NPs encapsulating the [fluorochrome](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;) 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR). Briefly, A549 tumor-bearing mice were aquired via injecting A549 cells (1×107/100 μL) subcutaneously into the right armpit of Barb/c nude mice. After nearly 3 weeks, mice were injected with DiR-loaded C-NPs and free DiR intravenously, and observed the distribution after 1, 4, 12 and 24 h. At the point of 24h, the mice were dissected to obtain the main tissues and imaged. Besides, the DiR intensity for each group was calculated to determine the accumulation of C-NPs. Data were processed followed by analyzing with Living Image 4.1 software.

**Curing Efficacy of Chemo-sonodynamic therapeutic**

The preparations were administrated to bilateral Barb/c nude mice models when tumor grown up to about 150-200 mm3. Soon afterwards, all mice were divided into 6 groups (n = 3) of normal saline (NS), C-NPs+SDT, DTX/C-NPs, DTX with Rh, DTX/C-NPs+SDT and Taxotere®. The mice were injected at alternate day starting from day 0 to day 8 (DTX dose of 10mg/kg). 4 h later, the SDT groups were treated by ultrasound (1.2 W/cm2, 3 min) only on the right armpit tumors. The tumor volumes were recorded and calculated every 2 days formula (width×width×lenght/2), and the body weight were also measured.

At the 14th day, all mice were deprived of food but water for 12 h, and the serum isolated from blood samples was collected by extirpating eyeball at day 15. The serum was used to test the biochemistry profiles (n=3) including alanine aminotransferase (ALT), aspartate amino-transferase (AST), blood urea nitrogen (BUN), and creatinine (CREA). Further, the serum expression of IL-10 and IL-12 were measured via mouse IL-10 ELISA kit as well as the mouse IL-12 (p70) ELISA kit (USA, BOSTER Biological Technology Co., Ltd).

Subsequently, the mice were executed followed by extracting tissues of heart, liver, spleen, lung and kidney. Later on, the tissues were embedded in paraffin, sliced for the purpose of hematoxylin and eosin (H&E) staining. To observe the tumor apoptosis, tumors in the bilateral mouse model were conducted with the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. Further, the expression of metastasis associated proteins like urokinase type plasminogen activator (uPA) as well as the cyclooxygenase 2 (COX-2) were determined through western blot assay in both two types of tumor tissues. In addition, the tumor tissues were frozen sectioned and stained with anti-CD31 and anti-CD 206 to observe the tumor vessels and expression of [macrophage](file:///C:/Users/dell/Desktop/Manuscript%20-2.docx#/javascript:;)s.

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