Supporting Information

Near-Infrared Conjugated Polymer for Photoacoustic Imaging-Guided Photothermal/Chemo Combination Therapy

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Experimental Section

Materials

Doxorubicin hydrochloride (DOX·HCl) was purchased from Energy-Chemical (Shanghai, China). 3,6-Bis(5-bromothiophen-2-yl)-2,5-bis(2-hexyldecyl) pyrrolo (3,4-c) pyrrole-1,4 (2H,5H)-dione and 2,5-Bis (trimethylstannyl) thiophene were

purchased from Suna Teck Inc (Suzhou, China). D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), Cholesteryl chloroformate (CHOCL), fetal bovine serum (FBS), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-Htetrazolium bromide (MTT), 4',6-Diamidino-2-phenylindole (DAPI) were purchased from Sigma Aldrich (St. Louis, MO, USA). Calcein-AM/PI Double Stain Kit was obstained from YEASEN (Shanghai, China). FITC Annexin V Apoptosis Detection Kit, BCA protein assay kit, cleaved caspase-3 antibody, caspase-3 antibody and ECL reagent were obtained from BD Biosciences (Franklin lakes, New Jersey, USA). HepG2 cell line was obtained from National Institute for the Control of Pharmaceutical and Biological Products. The solvents for chemical syntheses were purchased from commercial sources and purified by distillation.

Synthesis of DPP-based Conjugated polymer (DPP)

A 50 mL Schlenk tube was heated under reduced pressure and then allowed to cool to room temperature at nitrogen. 2,5-bis(trimethylstannyl)thiophene (123 mg, 0.3 mmol) 2,5-bis(4-bromobutyl)-3,6-bis(5-bromothiophen-2-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione(218 mg, 0.3 mmol) and dry chlorobenzene (15 mL) were added to this tube. The solution was degassed with argon for 30 min, followed by addition of $Pd_2(dba)_3$ (15 mg) and $P(o\text{-}tol)_3$ (60 mg) and then degassed and charged with argon several times. The reaction mixture was stirred vigorously at 120 °C for 72 h under argon. 2-Bromothiophene (30 mg, 0.2 mmol) was then added and the reaction was continued for another 12 h. After cooling down to 80 °C, aqueous solution of sodium diethyldithiocarbamatetrihydrate was added to remove the residual palladium catalyst.

After 12 h, the mixture was filtered and then extracted on a Soxhlet's extractor with acetone, hexane and chloroform successively. The final product was dried under reduced pressure at room temperature for at least 24 h to obtain a black solid, 204 mg, 85% yield. $(C_{26}H_{24}Br_2N_2O_2S_3)_n$.

Synthesis of tocopheryl polyethylene-glycol-succinate-cholesterol (TPGS-CHO) copolymer

15 g TPGS ($M_n \approx 1500$) and triethylamine (TEA) (1.2 mM, 0.2 mL) were dissolved in dry 20 mL of chloroform ($CHCl_3$) in a flask under N_2 protection. 4.5 g cholesteryl chloroformate (M_n =449.11) was dissolved in dry 10 mL of chloroform ($CHCl_3$) and added dropwise slowly into the solution. The reaction was stirred at 0 °C for 24 h. After removing $CHCl_3$, the solution was dialyzed against deionized water for 3 days. The product was collected by lyophilization.

Characterizations of NPs

Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured at room temperature by a Unity-400 MHz NMR spectrometer (Bruker). Diameters were performed with a Brookhaven 90Plus size analyzer. Transmission electron microscopy (TEM) images were recorded on a JEOL JEM-1011 electron microscope. UV-visible absorption spectra were recorded via a Varian Cary 300 UV-visible spectrophotometer in 1 cm path-length cuvette.

***In vitro* drug loading and release**

DOX-loaded TPGS-CHO nanoparticles (DOX-NPs), DPP-loaded TPGS-CHO nanoparticles (DPP-NPs) and coencapsulated TPGS-CHO nanoparticles (DOX/DPP-NPs) were prepared by a simple dialysis technique. Typically, DOX (10.0 mg), DPP-based polymer (5 mg) and TPGS-CHO (100.0 mg) and were mixed in 20 mL of THF. The mixture was stirred at room temperature for 24 h and then added dropwise into 100.0 mL of PBS at pH 7.4. The THF was removed by dialysis against water for 24 h. The dialysis medium was refreshed five times and the whole procedure was performed in the dark. Then, the solution was lyophilized. To determine the drug loading content (DLC) and drug loading efficiency (DLE), the drug-loaded NPs was dissolved in CH_2Cl_2 and analyzed by Uv-vis absorption measurement using a standard curve method. The DLC and DLE of drug-loaded NPs were calculated according to Eq. 1 and 2, respectively:

DLC (wt %) = amount of drug in nanoparticle /amount of drug loaded nanoparticle × 100% (1)

DLE (wt %) = amount of drug in nanoparticle /total amount of feeding drug × 100% (2)

In vitro drug release profile of DOX/DPP-NPs were investigated in PBS at 7.4. The pre-weighed freeze-dried DOX/DPP-NPs were suspended in 5.0 mL of release medium and transferred into a dialysis bag (MWCO 3500 Da). The release experiments were performed with and without 3 W cm^{-2} 808 nm lasers for 5 min at initial time of experiment at 37 °C. At predetermined time intervals, 2.0 mL of

external release medium was taken out and an equal volume of fresh release medium was replenished. The amount of released DOX was determined by using fluorescence measurement (λ_{ex} = 480 nm). The release experiments were conducted in triplicate.

Calculation of the photothermal conversion efficiency

Following Roper's report, the total energy balance for the system can be expressed by Eq. 3:

$$\Sigma m_i C_{p,j}\, dT/dt = Q_{NC} + Q_{dis} - Q_{surr} \quad (3)$$

where m and C_p are the mass and heat capacity of water, respectively. T is the solution temperature, Q_{NC} is the energy inputted by nanoparticles, Q_{Dis} is the baseline energy inputted by the sample cell, and Q_{Surr} is heat conduction away from the system surface by air.

The laser-induced source term, Q_{NC}, represents heat dissipated by electron-phonon relaxation of the plasmons on the nanoparticles surface under the irradiation of 808 nm laser:

$$Q_{NC} = I\,(1 - 10^{-A808})\,\eta \quad (4)$$

where I is incident laser power, η is the conversion efficiency from incident laser energy to thermal energy, and $A808$ is the absorbance of the nanoparticles at wavelength of 808 nm . In addition, source term, Q_{Dis}, expresses heat dissipated from light absorbed by the quartz sample cell itself, and it was measured independently to be 0.98 mW using a quartz cuvette cell containing pure water without nanoparticles.

Furthermore, Q_{Surr} is linear with temperature for the outgoing thermal energy, as given by Eq. 5:

$$Q_{Surr} = hS\,(T - T_{surr}) \tag{5}$$

where h is heat transfer coefficient, S is the surface area of the container, and T_{Surr} is ambient temperature of the surroundings. Once the laser power is defined, the heat input ($Q_{NC} + Q_{Dis}$) will be finite. Since the heat output (Q_{Surr}) is increased along with the increase of the temperature according to the Eq. 5, the system temperature will rise to the maximum when the heat input is equal to heat output:

$$Q_{NC} + Q_{Dis} = Q_{Surr\text{-}Max} = hS\,(T_{\max} - T_{surr}) \tag{6}$$

where the $Q_{Surr\text{-}Max}$ is heat conduction away from the system surface by air when the sample cell reaches the equilibrium temperature, and T_{max} is the equilibrium temperature. The 808 nm laser heat conversion efficiency (η) can be determined by substituting Eq. 4 for Q_{NC} into Eq. 6 and rearranging to get.

$$\eta = \frac{hS\,(T_{max} - T_{surr}) - Q_{Dis}}{I\,(1\text{-}10^{-A808})} \tag{7}$$

where Q_{Dis} was measured independently to be 0.466 W, the (T_{max} -T_{Surr}) were 31.9 °C from to Figure. 2C and 2D, I is 3 W/cm ^{2}, $A808$ is the absorbance (1.028) of nanoparticles at 808 nm. Thus, only the hS remains unknown for calculating η. In order to get the hS, a dimensionless driving force temperature, θ is introduced using the maximum system temperature, T_{max}

$$\theta = \frac{(T - T_{surr})}{(T_{max} - T_{surr})} \tag{8}$$

and a sample system time constant τ_s

$$\tau_s = \frac{\sum_i m_i C_{p,j}}{hS} \tag{9}$$

which is substituted into Eq. 1 and rearranged to yield

$$\frac{d\theta}{dt} = \frac{1}{\tau_s}\left(\frac{Q_{NC} + Q_{Dis}}{hS(T_{max} - T_{surr})} - \theta\right) \tag{10}$$

At the cooling stage of the aqueous dispersion of the nanoparticles, the light source was shut off, the $Q_{NC} + Q_{Dis} = 0$, reducing the Eq. 11

$$dt = -\tau_s \frac{d\theta}{dt} \tag{11}$$

and integrating, giving the expression

$$t = -\tau_s \mathrm{Ln}\theta \tag{12}$$

Therefore, time constant for heat transfer from the system is determined to be τ_s = 367 s by applying the linear time data from the cooling period vs negative natural logarithm of driving force temperature (Figure 2C and 2D). In addition, the *m* is 5 g and the *C* is 4.2 J/g. Thus, according to Eq. 9, the *hS* is deduced to be 0.057 W/°C substituting them into Eq. 7, the 808 nm laser heat conversion efficiency (η) of nanoparticles can be calculated to be 50.56%.

***In vitro* cellular uptake and chemo-photothermal treatment**

Cells were grown in DMEM culture medium containing 10% FBS, 1% penicillin and 1% streptomycin at 37°C under 5% CO_2. HepG2 cells were maintained in 6-well plates. After incubated for 24 h, the culture media were withdrawn and culture media containing DOX/DPP-NPs were supplemented to confirm that the final DOX concentration was 10 μg mL^{-1}. After incubation for 2 or 4 h, the cells were washed with PBS (0.01 M, pH 7.4) three times. Subsequently, the cells were stained with DAPI for 15 min. The cellular localization was visualized under a confocal laser scanning microscope (CLSM) (ZEISS LSM780, Germany). Flow cytometry was used to quantity study cell internalization of DOX. Internalization signals were analyzed by flow cytometer (BD Biosciences, Japan).

HepG2 cells (1×10^4 cells/well) were seeded into 96-well plate to quantitative evaluation of Chemo-photothermal Treatments efficacy of DOX/DPP-NPs. These cells were incubated in 200 μL of medium with different NPs but containing the same concentration of drug. The cells were irradiated with a 1.5 W cm^{-2} 808 nm laser for 5 min for chemo-photothermal treatments. For chemotherapy alone, the cells were not exposed to laser. After 48 h, the cell viability was quantified by the MTT assay.

To further visualize the cell cytotoxicity of chemothermal, photothermal, and chemophotothermal treatment, HepG2 cells were seeded into 24-well plates (5×10^4 cells/well) and incubated overnight. The cells were then replaced with fresh media containing different NPs. After 24 h of incubation, cells treated with DPP were exposed to 808 nm laser (1.5 W cm^{-2}). The group treated with PBS and laser irradiation were served as the control. After another 4 h, cells were washed with PBS

and stained with calcein-AM for visualization of live cells and with PI for visualization of dead/late apoptotic cells. The cells were examined by an inverted florescence microscope system (Olympus, Japan).

The cytotoxicity of chemo-photothermal treatment was also evaluated by quantification of apoptotic HepG2 cells. HepG2 cells were seeded at a density of 6×10^5 cells/well in 6-well plate and cultured overnight. Cells were treated with PBS, blank NPs, DPP-NPs and DPP-NPs + laser (3 W/cm^2, 5 min), respectively. To detect cell apoptosis, HepG2 cells were collected. Then the cells were stained by using an annexin V-FITC and PI taining kit following the manufacturer's instructions. The induction of apoptosis in HepG2 cells were examined by flow cytometer.

Total cellular proteins from cells treated with PBS+ laser, DOX-NPs, DPP-NPs + laser and DOX/DPP-NPs + laser were extracted by cell lysis buffer from cell signaling technology. The protein concentrations were determined by BCA protein assay kit. Effects of chemo-photothermal treatment on expression levels of apoptosis related proteins were determined by western blotting.

Animal Xenograft Model

Male BALB/C nude mice (4-6 weeks old, 18-22 g) were purchased from Center for Experimental Animals, Jilin University. The mice were treated under protocols approved by the School of Life Sciences Animal Care and Use Committee of Northeast Normal University. The HepG2 tumor models were successfully established by subcutaneous injection of 5×10^6 cells suspended in 100 μL PBS.

In Vivo Imaging and Biodistribution Analysis

BALB/C nude mice bearing HepG2 tumors were injected with DOX/DPP-NPs via tail vein. After 24 h, mice were irradiated by NIR laser (808 nm, 1 W cm^{-2}) for 5 min. The temperature change in tumor region under laser irradiation was recorded by an IR camera.

Photoacoustic imaging was performed on a MSOT scanner equipped with 128 ultrasound transducer elements (MSOT inVision 128, iThera Medical GmbH, Munich, Germany). Photoacoustic imaging of different times after injection was recorded by the MSOT system when the tumors were exposed to 808 nm laser light.

DOX and DOX-NPs were injected to tumor-bearing mice via tail vein with a dose of 3.0 mg kg^{-1} DOX equivalent. The mice were sacrificed at 2, 6 or 24 h post-injection. The major organs were excised immediately and subsequently washed with PBS three times for *ex vivo* imaging of DOX fluorescence on *in vivo* imaging system (Berthold, Germany).

In Vivo Antitumor Efficacy and Histology Analysis

The mice were directly injected via tail vein with different formulations (3.0 mg kg^{-1} DOX equivalent): (1) PBS; (2) PBS + Laser; (3) blank NPs; (4) Free DOX; (5) DOX-NPs; (6) DOX/DPP-NPs; (7) DPP-NPs + Laser; (8) DOX-DPP-NPs + Laser. 24 h after injection, laser was conducted. For the laser treatment groups, the tumors of mice were exposed to 808 nm laser of power density at 1 W cm^{-2} for 5 min. Twice

injections were performed on the first day and fifth day. Quantitative changes in tumor volume and body weight of mice were measured. Tumor volume was calculated as length × width^2/2.

To further confirm the effect of mediated chemo-photothermal therapy *in vivo*, tumor tissues at 2 h and the major organs (liver, lung, kidney, heart, and spleen) at 20 d after first treatment were stained with hematoxylin and eosin.