Supporting Information

Light-Triggered Release of Drug Conjugates for Efficient Combination of Chemotherapy and Photodynamic Therapy

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1. Materials and methods

1.1 Reagents and materials

Dextran (Dex, \(M_n = 40\) kDa), 3-mercaptopropionic acid, 4-dimethylaminopyridine (DMAP), \(N\)-(3-dimethylaminopropyl)\(N\)'-ethylcarbodiimide hydrochloride (EDC•HCl), \(N\)-hydroxysuccinimide(NHS), succinic anhydride (SA), 3-(4,5)-dimethylthiahiazol (-z-yl)-3,5-di-phenytetrazoliumromide (MTT) were purchased from Sigma-Aldrich. Doxorubicin hydrochloride (DOX•HCl) was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd. The other reagents and solvents were purchased from Sinopharm Chemical Reagent Co, Ltd.

1.2 Characterizations

\(^1\)H NMR spectra were recorded on a Bruker AV 400 NMR spectrometer in dimethyl sulfoxide-\(d_6\) (DMSO-\(d_6\)). Fourier Transform infrared (FTIR) spectra
were recorded using a Bio-Rad Win-IR instrument. The sizes and distributions of particles were tested by a WyattQELS dynamic laser scattering (DLS) instrument with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology). Transmission electron microscopy (TEM) images were taken by a JEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 Kv. Flow cytometry (Coulter Epice XL, Beckman Coulter, USA) was performed to quantitatively determine the percentage of cells. Confocal laser scanning microscopy (CLSM) images were captured on Olympus FluoView 1000. Bio-Rad 680 microplate reader was used to conduct MTT.

1.3 Synthesis of 5-(4-aminophenyl)-10,15,20-triphenylporphyrin (Por-NH$_2$)

Por-NH$_2$ was prepared as following$^{1, 2}$.

Firstly, meso-tetraphenylporphyrin (TPP) was prepared as following. Freshly distilled pyrrole (80 mM, 5.6 mL) and benzaldehyde (80 mM, 8 mL) were added into propionic acid (300 mL). After refluxing for 3 min, the solution was cooled to room temperature and filtered; the filter cake was washed with methanol. After washing with hot water, the given purple crystals were dried in a vacuum oven to remove absorbed water. Finally, the crude product was purified by recrystallization using dichloromethane (CHCl$_2$) and methanol.

Secondly, sodium nitrite (100 mg, 1.45 mmol) was added to TPP (500 mg, 0.816 mmol) solution in TFA (50 mL) with stirring at room temperature for 3 min. The reaction was quenched with water, and the mixture was extracted with dichloromethane (CH$_2$Cl$_2$). The organic layers were washed with saturated aqueous NaHCO$_3$ and water, then dried over anhydrous Na$_2$SO$_4$. The obtained mono nitro porphyrin was recrystallized from CH$_2$Cl$_2$. And then, mono nitro porphyrin (500 mg, 0.815 mmol) was dissolved in hydrochloric acid (300 mL) and tin (II) chloride (220 mg, 0.97 mmol) was added into the solution. The mixture was heated to 65 °C and stirred for 1 h under argon before pouring into cold water (400 mL). The aqueous solution was neutralized with ammonium hydroxide until pH 8. Then the aqueous solution was extracted with dichloromethane until colorless. The organic layer was concentrated under vacuum, and the residue purified on a plug of alumina using dichloromethane for elution. The obtained residue was recrystallized from petroleum ether.
1.4 Synthesis of succinic anhydride modified Por-NH$_2$ (Por-SA)

Por-SA was synthesized through the ring-opening reaction between Por-NH$_2$ and SA with DMAP as catalyst. Por-NH$_2$ (100mg, 0.159 mmol) and SA (31.8 mg, 0.318 mmol) were dissolved in anhydrous DMF (10.0 mL) in a dried flask, and then DMAP (19.4 mg, 0.159 mmol) was added. The mixture was stirred under a nitrogen environment at 45 °C for 24 h. After that, the solution was mixed with 100 mL of cold ethyl acetate, then the mixture was washed with cold acidic saturated sodium chloride solution (pH 2-3) and finally with normal pH 7.4 saturated solution. The organic layer was collected and dried with anhydrous sodium sulfate overnight. After filtration, the filtrate was dried under vacuum at room temperature to obtain product.

1.5 Synthesis of ROS-cleavable linker modified dextran (DEX-TK).

We first synthesized ROS-cleavable thioketallinker (TK). A mixture of anhydrous 3-mercaptopropionic acid (2 g, 18.9 mmol) and anhydrous acetone (2.23 g, 37.8 mmol) were saturated with dry hydrogen chloride and stirred at room temperature for around 6 h. Then the reaction was chilled in an ice-salt mixture to realize completely crystallization. The crystals were filtered, washed with hexane and cold water, the product was finally obtained after drying in a vacuum desiccator.

To prepare TK modified dextran, dextran (100 mg, 0.167 mmol) was dissolved in DMSO, and then TK (77.8 mg, 0.3 mmol), EDC•HCl (177.5 mg, 0.926 mmol) and DMAP (22.6 mg, 0.185 mmol) were added into the solution with stirring at room temperature for 48 h. After that, the mixture was dialyzed against deionized water water for 3 days to remove the solvent and unreacted substances. The product was collected by freeze-drying under vacuum, finally obtained white powders.

1.6 Synthesis of Por-SA-DEX-TK-DOX polymeric conjugates

Por-SA-DEX-TK-DOX conjugates were fabricated through the simple condensation reaction.

The carboxyl group of DEX-TK was conjugated with the amine group of DOX under the catalysis of EDC and NHS. DEX-TK (100 mg, 0.347 mmol),
doxorubicin (200 mg, 0.347 mmol) and triethylamine (100 µL, 0.717 µmol) were dissolved in DMSO, then EDC (199.6 mg, 1.04 mmol) and NHS (119.8 mg, 1.04 mmol) were added into the above solution. The reaction was stirred at room temperature for 48 h. After that, the solvent and unreacted substances were removed by dialysis against deionized water for 3 d. The solution was lyophilized to obtain the red powders product.

Por-SA was conjugated on dextran backbone through the reaction between the hydroxyl group of DEX-TK-DOX and the carboxyl group under the catalysis of EDC and DMAP. Briefly, DEX-TK-DOX (100 mg, 0.216 mmol) and Por-SA (23 mg, 0.032 mmol) were dissolved in DMSO, then EDC (18.7 mg, 0.097 mmol) and DMAP (2.4 mg, 0.019 mmol) were dissolved in DMSO and added into the above solution. The mixture was stirred at room temperature for 48 h, after that, the solvent and unreacted substances were removed by dialysis against deionized water for 3 days. Finally, the product was obtained by lyophilization under vacuum.

1.7 In vitro drug release

Por-SA-DEX-TK-DOX conjugates were dissolved in DMSO and analyzed by fluorescence measurements (Perkin-Elmer LS50B luminescence spectrometer). A standard curve (λ_{ex} = 480 nm) was obtained from different DOX concentrations. The conjugated ratios were calculated according to the following Equations:

Conjugated ratio (wt %) = amount of DOX in conjugates / amount of DOX-conjugates × 100 (1)

In vitro DOX release behavior from Por-SA-DEX-TK-DOX conjugates were investigated in PBS at 7.4. The pre-weighted lyophilized DOX-loaded nanoparticles were suspended in 3 mL of release media, and then transferred into a dialysis tubes (MWCO = 3500 Da). The release experiment was initiated by placing the end-sealed dialysis bag into 50 mL of PBS at 37 °C with continuous shaking at around 1000 rpm. At definite time intervals, 2.0mL of dialysate was taken out and an equal volume of fresh buffer was added. At the
second, 12th and 24th hours, the dialysis bag included the samples were took out and applied light irradiation for 5 mins (40 mW/cm², 400-700 nm), and then put into the PBS solution to continue the release experiment. The amount of release DOX was measured by fluorescence measurement (λ_{ex}= 480nm). The release experiments were conducted in triplicate.

1.8 Intracellular drug release

The cellular uptake and intracellular release of DOX-conjugates were analyzed by confocal laser scanning microscopy (CLSM) and flow cytometric analyses on HeLa cells.

Confocal laser scanning microscopy (CLSM)

For this study, HeLa cells were seeded in six-well plates at a density of 2 × 10⁵ cells per well in 2.0 mL of complete DMEM (Dulbecco’s modified Eagle’s medium) containing 10 % fetal bovine serum, supplemented with 50 IU mL⁻¹ penicillin and 50 IU mL⁻¹ streptomycin. After incubation for 24 h, the culture media were withdrawn and the cells were incubated with DOX-conjugates at a final DOX concentration of 10.0 mg L⁻¹ in complete DMEM. After treating by light irradiation for 10 mins (40 mW/cm², 400-700 nm), the sample were incubated for additional 6 h at 37 °C. Then the culture medium was removed and the cells were washed with PBS three times. Thereafter, the cells were fixed with 4 % paraformaldehyde for 30 min at room temperature, and the cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, blue) for 20 min. The images of cells were obtained through confocal microscope (Olympus FluoView 1000).

Flow cytometric analyses

HeLa cells were seeded into 6-well plates at 2 × 10⁵ cells per well in 2.0 mL of complete DMEM and cultured for 24 h. The cells were incubated with DOX-conjugates at a final DOX concentration of 10.0 mg L⁻¹. After treating by 10 min
light irradiation at 37 °C (40 mW/cm², 400-700 nm) and incubated for additional 6 h, the culture medium was removed and the cells were washed with PBS thrice and treated with trypsin. Then, 2.0 mL of PBS was added to each culture well, and the solutions were centrifuged for 4 min at 3000 rpm. After the removal of supernatants, the cells were re-suspended in 0.3 mL of PBS. Data for \(1 \times 10^4\) gated events were collected, and analysis was performed by flow cytometer (Beckman, California, USA).

**Cell viability assays**

The cytotoxicity of DOX conjugates against HeLa cells were evaluated *in vitro* by a standard MTT assay. The cells were seeded into 96-well plates at \(1 \times 10^4\) cells per well in 200.0 \(\mu\)L of complete DMEM and further incubated at 37 °C for 24 h. Then washed cells with PBS, 180.0 \(\mu\)L of complete DMEM and 20.0 \(\mu\)L of DOX conjugates solutions in PBS were added to form culture media of different DOX concentrations (0-10.0 mg L\(^{-1}\) DOX). After treating by 10 min light irradiation at 37 °C (40 mW/cm², 400-700 nm) and incubated for additional 6 h, the cells were subjected to MTT assay after incubation for 24 h and 48 h. The absorbance was measured on a Bio-Rad 680 microplate reader at 490 nm. Cell viability (%) was calculated based on eqn (3).

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\text{Cell viability (\%) = } \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100
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where, \(A_{\text{sample}}\) and \(A_{\text{control}}\) represent the absorbance of the sample and control wells, respectively.

**1.9 The measurement of singlet oxygen (\(^1\)O\(_2\))**

Generation of singlet oxygen can be usually detected by chemical method using 9,10-anthracenediy-l-bis (methylene) dimalonic acid (ABDA) as a kind of singlet oxygen sensor. ABDA was chosen to monitor the release of singlet oxygen into solution by recording the decrease in absorption of ABDA at 376 nm via UV-vis spectroscopy. Briefly, 50\(\mu\)L of ABDA solution (1 g/mL) was added into 2mL PSDTD solution of PBS (0.5g/mL). Absorption intensity of ABDA at 376 nm was
monitored after different time light illumination\(^4\) (40 mW/cm\(^2\), 400-700 nm).

\textbf{1.10 Intracellular ROS Measurement.}

The intracellular ROS detection was conducted as in previous report\(^5\). Shortly, HeLa cells were cultured in six-well plates at a density of \(2 \times 10^5\) cells per well in 2.0 mL of complete DMEM (Dulbecco’s modified Eagle’s medium) containing 10 % fetal bovine serum, supplemented with 50 IU mL\(^{-1}\) penicillin and 50 IU mL\(^{-1}\) streptomycin. After incubation for 24 h, the culture media were withdrawn and the cells were incubated with DOX-conjugates at a final DOX concentration of 10.0 mg L\(^{-1}\) in complete DMEM. Cells were repeatedly washed with PBS buffer after incubation for 6h at 37 °C. Adding DMEM supplemented with DCFH-DA (ROS sensor, final concentration: \(1 \times 10^{-5}\) M). Twenty minutes later, cells were washed with PBS buffer thoroughly to remove residual DCFH-DA and received 20 min of light irradiation. The image of cells were obtained through confocal microscope (Olympus FluoView 1000).
Scheme S1 The synthesis route of Por-SA.

Scheme S2 The synthesis route and structure of DEX-TK-DOX.
Scheme S3 The synthesis route of and structure of Por-SA-Dex-TK-DOX.

Fig. S1 $^1$H NMR spectrum of Por-NH$_2$ and Por-SA in CDCl$_3$. 
Fig. S2 FTIR spectra of Por-SA.

Fig. S3 $^1$H NMR spectra of TK in CD$_3$OD.

Fig. S4 $^1$H NMR spectra of Dex-TK.
Fig. S5 FTIR spectra of Dex-TK.

Fig. S6 $^1$H NMR spectra of Dex-TK-DOX.
Fig. S7 $^1$H NMR spectra of Por-SA-DEX-TK-DOX.

Fig. S8 FTIR spectra of Por-SA-Dex-TK-DOX.
**Fig. S9** *In vitro* light triggered DOX release profiles for PSDTD-1 at 37 °C with or without light irradiation.

**Fig. S10** *In vitro* light triggered DOX release profiles for PSDTD-1 at 37 °C with or without light irradiation.
**Fig. S11** Photo-bleaching of ABDA at PBS buffer solution (ABDA absorption at 376 nm) generated by singlet oxygen.

**Fig. S12** Photo-bleaching of ABDA (ABDA absorption at 376 nm) by singlet oxygen generated by DOX-conjugated nanoparticle (0.5mg/mL) in PBS.

**Fig. S13** Decay curves of ABDA absorption at 376 nm as a function of illumination time, corresponding to ABDA alone (a) and the mixture of ABDA and DOX-conjugated nanoparticles (b)
**Fig. S14** Representative CLSM images of HeLa cells with PSDTD-1 under light irradiation (A) and without light irradiation (B). For each panel, the images from left to right show differential interference contrast (DIC) images, cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), and overlays of the three images.

**Fig. S15** Representative CLSM images of HeLa cells with PSDTD-2 under light irradiation (A) and without light irradiation (B). For each panel, the images from left to right show differential interference contrast (DIC) images, cell nuclei stained by DAPI (blue), DOX fluorescence in cells (red), and overlays of the three images.
**Fig. S16** Flow cytometry profiles of HeLa cells incubated with PSDTD-1 without light irradiation (a) and PSDTD-1 under light irradiation (b).

**Fig. S17** Flow cytometry profiles of HeLa cells incubated with PSDTD-2 without light irradiation (a) and PSDTD-2 under light irradiation (b).
**Fig. S18** Flow cytometry profiles of HeLa cells incubated with PSDTD-3 without light irradiation (a) and PSDTD-3 under light irradiation (b).

**Fig. S19** Cytotoxicity of PSDTD-1 (A) and PSDTD-2 (B) toward HeLa cells after incubation for 24 h/48 h with and without light irradiation compared with free DOX.
Reference