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Hypoxia-Responsive Bilirubin Supramolecular Nanoprodrugs for Targeted Photothermal-Chemotherapy

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Materials and methods

Materials

Bilirubin (BR, 97%, Alfa), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 98%, Adamas), N-hydroxysuccinimide (NHS, 98%, Adamas), triethylamine (Et_3N, 99.5%, Adamas), 4-di-N-oxide tirapazamine (TPZ, 98%, Aladdin) were purchased. The pillar[5]arene-terminal- modified poly($_L$-lysine) (P5-PLL) and pyridinium-terminal-modified poly($_L$-lysine) (P-PLL) were synthesized following the procedure described previously.[1]

Synthesis of PPLAC

Briefly, P5-PLL (20 mg, 0.0224 mmol) was added to 10.0 mL dried MeOH with lactonolactone (192.4 mg, 0.5654 mmol) and Et_3N (83.5 μL, 0.6 mmol). After vigorous stirring for 48 h at 50 ^oC. The resulting solution was dialyzed for 48 h to remove excess small molecules in the mixture, and then lyophilized to obtain target product PPLAC (120.1 mg, 82.5% yield).

Synthesis of PBR

Bilirubin (58.5 mg, 0.1 mmol), EDC (23.6 mg, 0.15 mmol) and NHS (6.9 mg, 0.06 mmol) were dissolved in 5 mL dimethyl sulfoxide (DMSO). After stirring at room temperature for 10 minutes, P-PLL (20.68 mg, 0.005 mmol) was added and stirred under nitrogen atmosphere for 4 h. Then, 200 mL ethyl acetate was added to remove the free BR. The precipitate was filtered, and successively washed with 0.1 M HCl and ethanol. After vacuum drying, we successfully got PBR (39.2 mg, 61.4% yield).

Fabrication of bilirubin supramolecular nanoprodrug SCBR/TPZ

Firstly, PBR (5 mg) and TPZ (1.0 mg) were dissolved in 1.0 mL tetrahydrofuran, and then stirred 6 h in the dark under a stream of nitrogen gas. Secondly, 10 mL aqueous solution of PPLAC (3.16 mg) was slowly added into the original solution, and kept stirring for overnight. Finally, the mixture solution was transferred to a dialysis tube (MWCO 1000 Da) to purify by dialysis against distilled water. The finally obtained nanoprodrug was denoted as SCBR/TPZ.

Evaluation of O_2-scavenging effect of SCBR/TPZ

To examine the scavenging effect of SCBR/TPZ toward O_2, the dissolved oxygen of SCBR/TPZ and distilled water was measured using dissolved oxygen measurement.

***In vitro* drug release**

Generally, dialysis bag contained of SCBR/TPZ in PBS (1 mg/mL) was dipped in PBS containing DMSO (1 M) with or without addition of 50 μM H_2O_2 at 37 ^oC. The original dialysate was replaced with new dialysate at selected time intervals. For the irradiation groups, were irradiated with NIR irradiation (808 nm, 1 W/cm^2, 5 min) was used to irradiate the samples at predetermined time. The amount of released was quantified by UV-Vis spectroscopy at 460 nm. The TPZ release under hypoxic condition was quantified as control by using deoxygenated PBS solution.

***In vitro* cytotoxicity**

HepG2 cells were seeded in 96-well plate at a density of 1 × 10^4 cells/well. After incubating for 12 h, the fresh DMEM containing varied concentrations of free TPZ, SBR/TPZ or SCBR/TPZ replaced the original medium. After 4 h incubation, the irradiation groups were irradiated with NIR irradiation (808 nm, 1 W/cm^2, 5 min) and

incubated for another 48 h. The cytotoxicity was analyzed by using MTT assay.

TPZ serves as a substrate for one-electron ($1e^-$) reductases. The resultant free radical (TPZ•) undergoes spontaneous decay to either an oxidizing hydroxyl radical (OH•) or an oxidizing benzotriazinyl radical (BTZ•). The available evidence indicates that the double-strand breaks are not directly caused by the oxidizing radical (OH• or BTZ•), but, at least in part, through the poisoning of topoisomerase II. This could stem from the radical damage directly inflicted on the topoisomerase II enzyme, thereby poisoning it in the middle of its catalytic cycle and resulting in a double-strand break in a manner similar to etoposide; or the radical damage to DNA could act as a substrate for topoisomerase II, thus giving rise to double-strand breaks.[2-4]

Cell internalization and intracellular hypoxia

The HepG2 cells were plated in a 12-well dish at a concentration of 1.0×10^5 cells/well, and cultured for 12 h. Subsequently, the culture medium was substituted with fresh DMEM containing SCBR/TPZ or SCBR/TPZ (15 μg/mL TPZ equiv.). After incubating for 4 hours, the cells were treated with DAPI or hypoxia reagent for 20 minutes and then observed using a confocal laser scanning microscope (CLSM).

***In vivo* biodistribution**

The experimental protocol was approved by the Nantong University Institutional Animal Care and Use Committee. Furthermore, all animal experiments followed the institutional and national guidelines and were approved by the appropriate institutional review. The HepG2 tumors (~100 mm^3) bearing nude mice were randomly divided into three groups (n = 3), and intravenously injected with SBR/Cy7 or SCBR/Cy7 at a Cy7

dose of 1 mg/kg. Then, the *in vivo* tumor accumulation and biodistribution of was detected by a Kodak multimode imaging system at selected time intervals. In addition, the major organs and tumors were collected at 8 h post-injection, and imaged for *ex vivo* distribution.

***In vivo* antitumor activity**

The HepG2 tumors (70-90 mm^3) bearing nude mice were randomly divided into five groups (n = 4), and intravenously injected with PBS, free TPZ, free BR + L, SBR/TPZ + L, SCBR/TPZ, or SCBR/TPZ + L at a TPZ dose of 5 mg/kg on day 0 and day 4. As for the irradiation groups, the tumor tissue was irradiated with NIR irradiation (808 nm, 1 W/cm^2, 5 min) 8 h post-injection. The tumor volume and body weight were monitored every 2 days. The formula used to calculate the tumor inhibitory rates (TIR) is as follows: TIR (%) = 100 × (mean V of PBS group - mean V of others)/(mean V of PBS group).

Statistical analysis

The data were presented as mean ± SD, and the statistical significance of the differences between groups was assessed using Student's t-test.

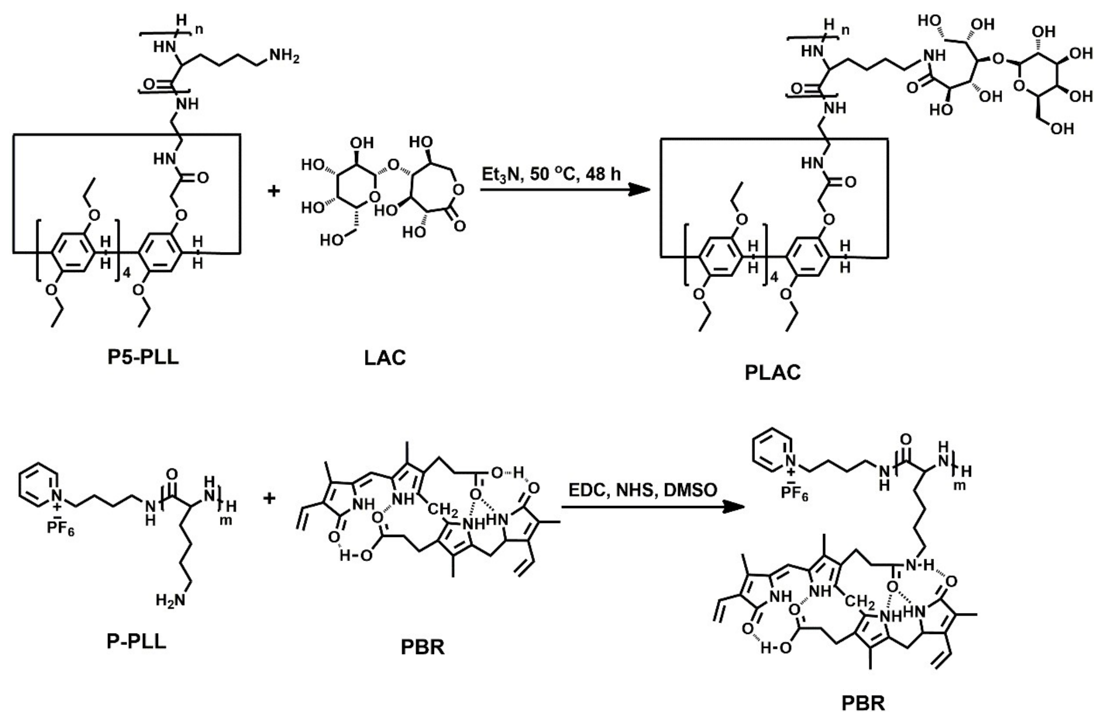

Fig. S1. Synthesis of PLAC and PBR.

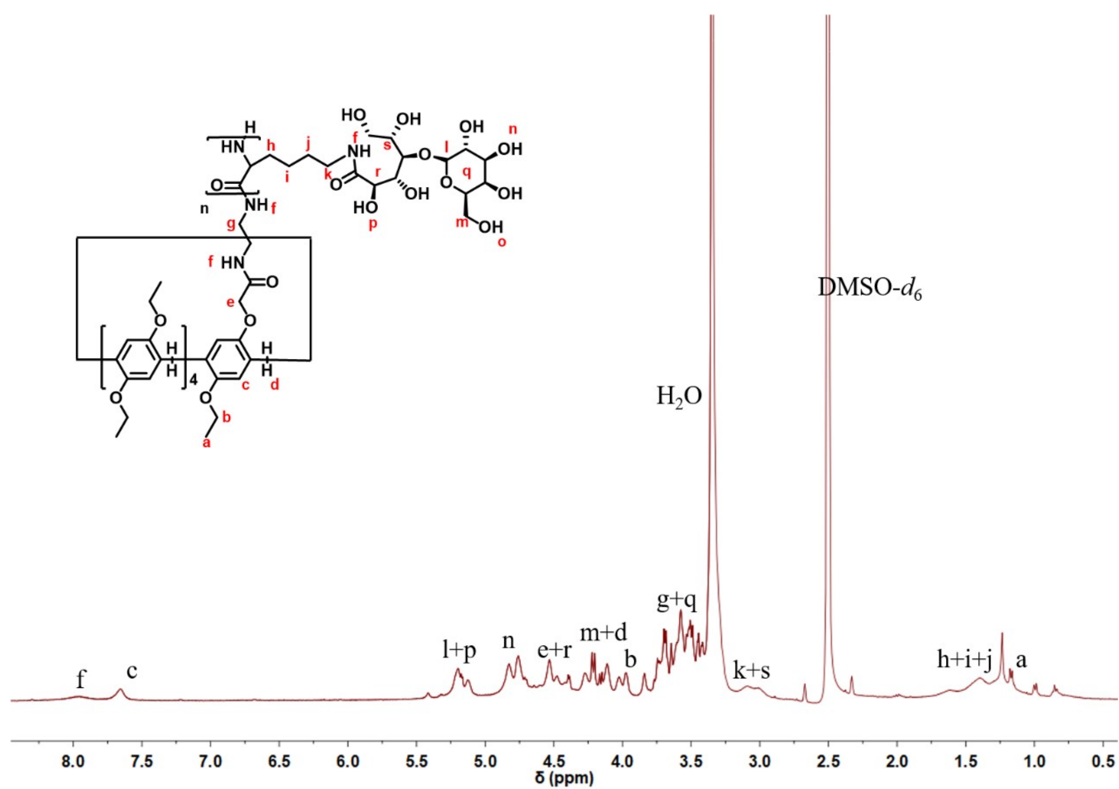

Fig. S2. ^{1}H NMR spectra of PLAC (DMSO-d_6).

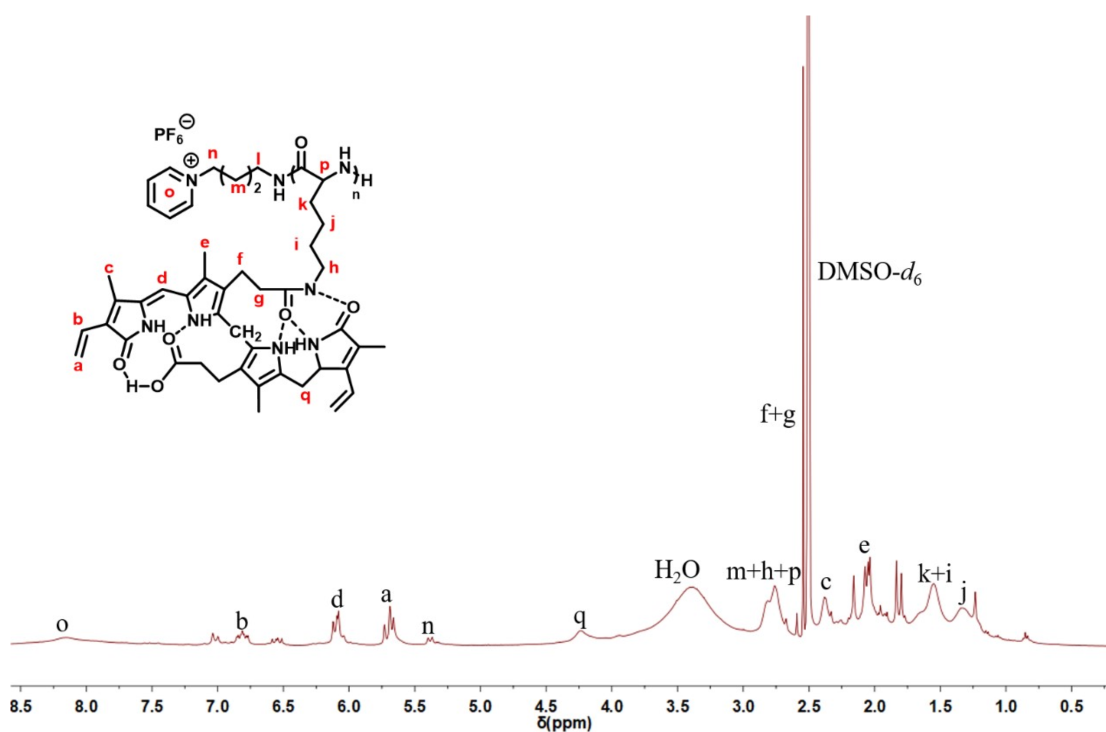

Fig. S3. ^{1}H NMR spectra of PBR (DMSO-d_6).

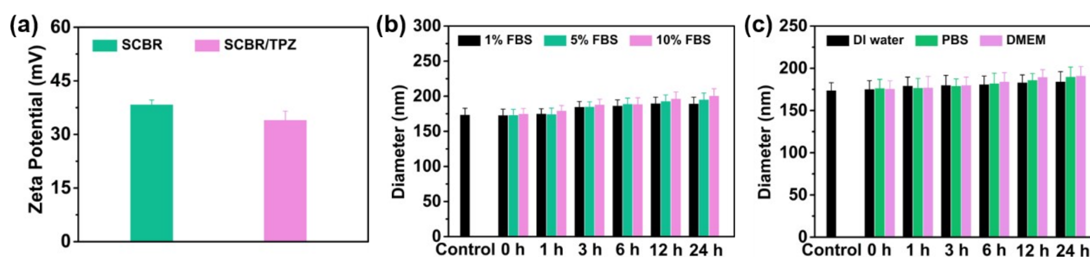

Fig. S4. (a) Zeta potential of SCBR and SCBR/TPZ. The hydrodynamic diameter of SCBR/TPZ incubated with FBS at different concentrations (b), DI water, PBS, and DMEM (c).

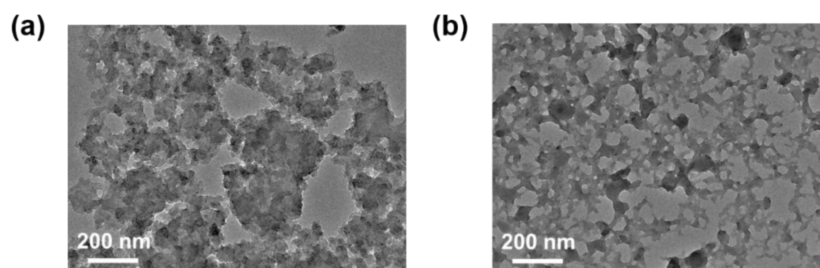

Fig. S5. TEM images of SCBR/TPZ at 24 under normoxic condition without (a) or with (b) adding H_2O_2.

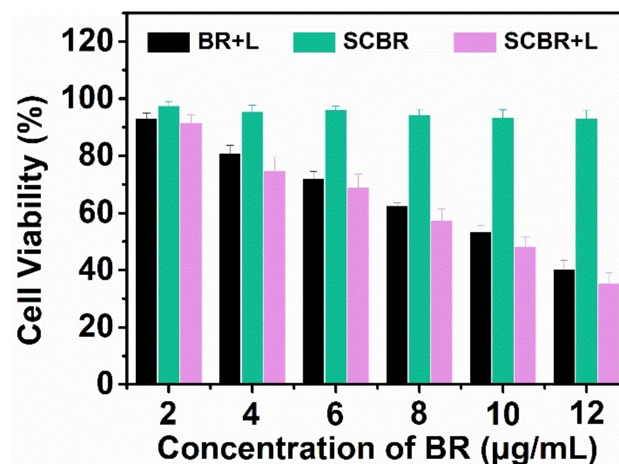

Fig. S6. In vitro cytocompatibility of BR and SCBR against HepG2 cells after 48 h incubation.

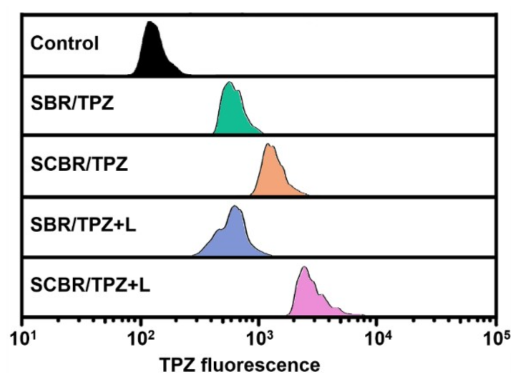

Fig. S7. Flow cytometry histograms of SBR/TPZ, SCBR/TPZ, SBR/TPZ+L, SCBR/TPZ+L.

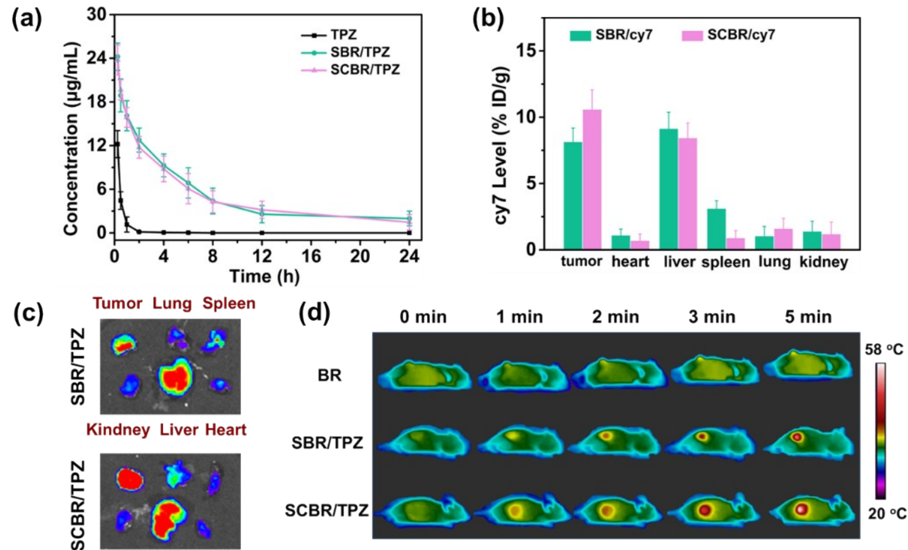

Fig. S8. (a) Pharmacokinetics profiles of TPZ, SBR/TPZ, and SCBR/TPZ. Ex vivo biodistributions (b) and imaging (c) of SBR/cy7 and SCBR/cy7 group in tumor tissue and major organs at 8 h. (d) The photothermal images of HepG2 tumor over the irradiation time.

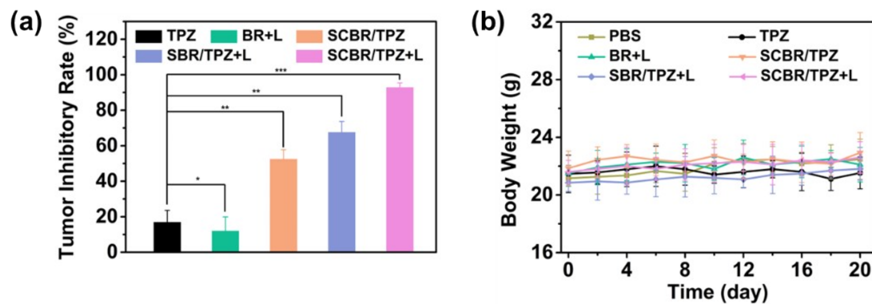

Fig. S9. (a) TIR for various treatments. $^{**}P < 0.01$, and $^{***}P < 0.001$. (b) Body weight changes of mice on time.

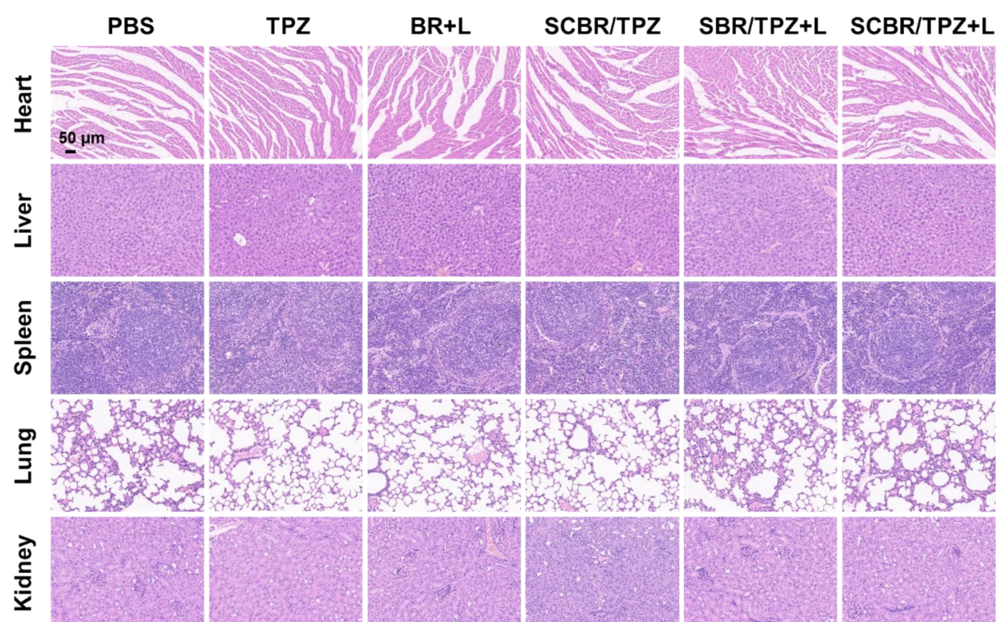

Fig. S10. H&E-stained tissue sections from the major organs (heart, liver, spleen, lung, and kidney) dissected after 20 days treatments (magnification × 400).

References

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