Electronic Supplementary Information

Targeting and Structural Engineering of light-responsive nanoprobe for Hierarchical Therapy: Construction, Optimization, and Applications in Cancer Stem Cells

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***In vitro* experiment**

Cell Culture

Human colon cancer cell line (SW-620) was obtained from the ATCC, and colon cancer stem cells (CCSCs) were sorted from SW-620 cells using CD133 (Prominin-1) monoclonal antibody as a marker. High glucose Dulbecco's modified Eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin−streptomycin, fetal bovine serum, phosphate-buffered solution (PBS), and streptomycin were purchased from commercial sources.

Human colorectal cancer cells (SW620) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The cells were maintained at 37 °C in a 5% CO_2 humidified incubator.

CCSCs were cultured in a serum-free medium in a low-adherence culture flask supplemented with epidermal growth factor and leukemia inhibitory factor. The culture medium was further enriched with a 1% antibiotic-antimycotic formulation. Incubation of cells took place in a 5% CO_2 incubator at 37 °C.

Cell internalization mechanism

To investigate the cellular uptake mechanism of Hb-AHSNs nanoparticles in SW620 cells, cells were seeded in a 96-well plate at a density of 5×10^3 cells per well. After a 24-hour incubation in serum-free DMEM, cells were treated for 2 hours at 4 °C or in the presence of specific inhibitors, including 20.2 μg/mL of genistein, 8 μg/mL amiloride, 6.5 mg/mL M-β-CD, 3 mM of chlorpromazine, 8 mM of NaN_3, 20 μg/mL dextran sulfate, respectively. Subsequently, 10 μg/mL of Hb-AHSNs nanoparticles were added to each group and incubated for 2 hours. Cellular fluorescence intensity was assessed using flow cytometry.

PTT/PDT treatment

Cells were seeded in 6-well culture plates and incubated for 24 hours. Subsequently, the cells were treated with different concentrations of nanoparticles in the dark for 24 hours (unless stated otherwise) and then washed with PBS. Before photodynamic therapy (PDT), the complete culture medium was fully replaced. Cells were treated

with or without laser irradiation (808 nm for PTT, 590 nm for PDT, 0.5 W/cm^2) for different time.

To investigate the synergistic effect of PDT-PTT-photothermal combination cancer therapy, typically, Hb-AHSNs-Ab (50 μg/mL) were added to the cells and incubated for 2 h. Then, cells were irradiated by 808 nm and 590 nm laser (0.5 W/cm^2) for 5 min sequentially. The cells were further incubated for 24 h, and viability was measured using CCK-8 assay and expressed relative to the non-treated, laser irradiated cells. The single PDT effect of AHSNs or Hb-AHSNs was evaluated by comparison with the results of treatment with an equivalent amount of Hyp-TAT. For quantitative analysis, synergistic factors were calculated by dividing the predicted additive viability by the viability of combination therapy. Synergistic factor >1 indicates synergistically enhanced cell killing by the combination treatment compared to the sequential treatment of the individual modalities.[1]

Cytotoxicity Assay

Cells were seeded in 96-well culture plates and treated according to the aforementioned protocol. MTT solution (20 μL, 5 mg/mL) was added to each well, and the cells were incubated for 4 hours in the dark. Subsequently, the formazan crystals were dissolved with 150 μL of DMSO. Absorbance was measured at 490 nm using a microplate reader. The results were expressed as percentages relative to the absorbance of the untreated control. The half inhibition concentration (IC50) was determined based on the relative survival curve.

Reactive oxygen species measurement

The evaluation of intracellular oxidative stress was conducted using the Reactive Oxygen Species Assay Kit, incorporating DCFH-DA. Following nanoparticles incubation, cells were washed twice with PBS. Subsequently, 1 mL of DCFH-DA solution (10 μM) was added, and the cells were incubated at 37 °C for 20 min. The supernatant was aspirated, and the cells were washed another two times with PBS. The cellular response was captured using a fluorescence microscope.

Immunofluorescence

Cells treated with PDT or PTT were fixed using immunostaining fix solution (for 20

min, followed by permeabilization with immunostaining permeabilization buffer or 10 min. Subsequently, cells were blocked with 3% BSA in PBS for 1.5 hours at room temperature. For immunofluorescence analysis, CDC25A and CDK2 primary antibodies were applied and allowed to incubate overnight at 4 °C. FITC-conjugated secondary antibody was used to incubate the cells for 45 min at room temperature. The nuclei were stained with Hoechst 33342 for 10 min. The cells underwent three washes and were subsequently observed and observed using a fluorescence microscope.

Combination therapy for CCSCs-related tumorsphere formation

Primary tumor (SW620) cells were dissociated into single cells and seeded in ultralow attachment 6-well plates at a density of 2000 cells/well. Subsequently, Hb-AHSNs or Hb-AHSNs-Ab were added to the cells (15 μg/mL), and incubate for 24 hours, followed by PDT-PTT treatment administered once every six hours, totaling three treatments. Stem cell frequency was assessed for each group. The cells were then cultured for 10 days, and tumorspheres larger than 50 μm in diameter were counted. The stem cell frequency of each group was reassessed.

Statistical Analysis

The data were expressed as means ± standard deviation (S.D.), and each experiment was performed in triplicate. Statistical analysis was conducted using an unpaired Student's t-test. A significance level of $P<0.05$ was considered statistically significant. The significance levels were denoted as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$, and "ns" indicated no significant differences from the control.

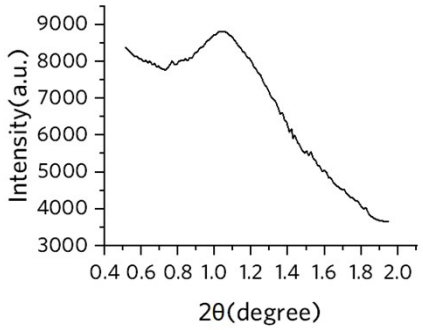

Fig. S1 (a) Small-angle x-ray scattering result of the obtained SNs.

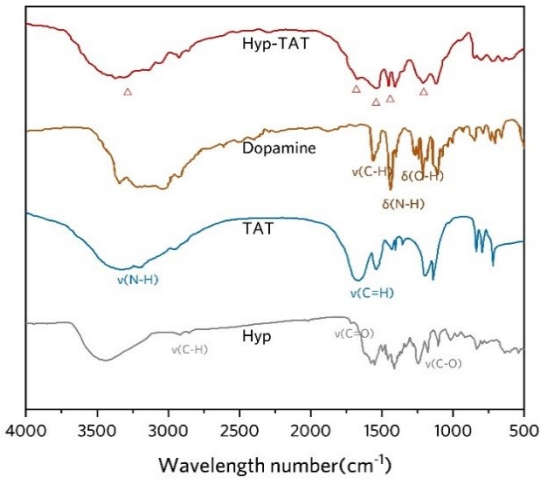

Fig. S2 FTIR spectra of hypericin, TAT, dopamine and Hyp-TAT, respectively.

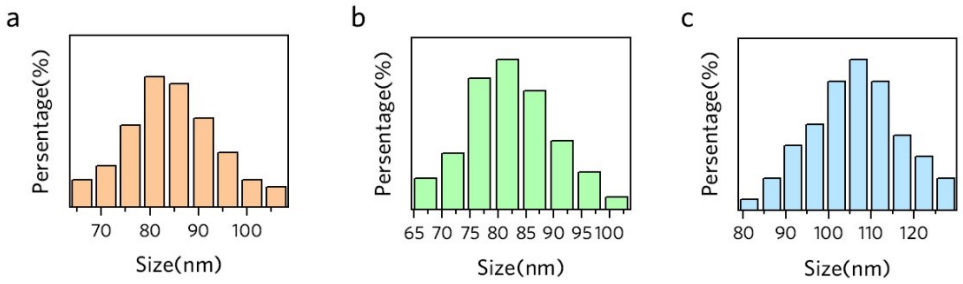

Fig. S3 Histograms of size distribution of SNs (a), HSNs (b) and AHSNs (c), respectively.

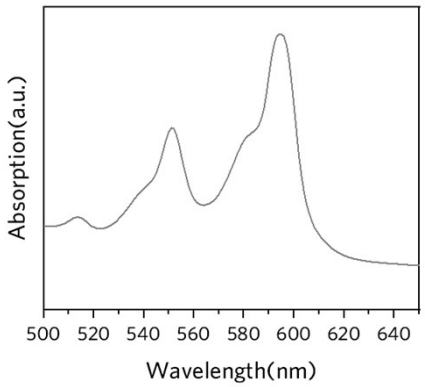

Fig. S4 Absorption spectrum of hypericin.

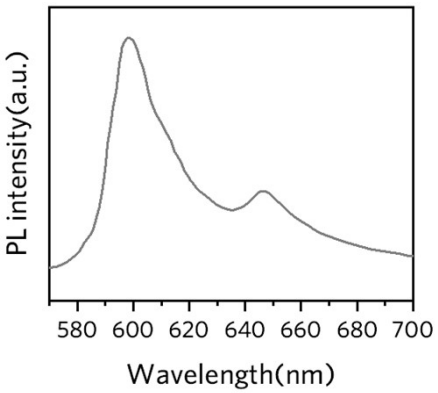

Fig. S5 PL spectrum of hypericin.

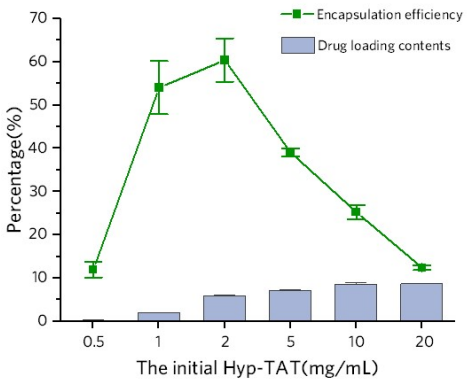

Fig. S6 Encapsulation efficiency and drug loading contents of SNs under different initial concentrations of Hyp-TAT.

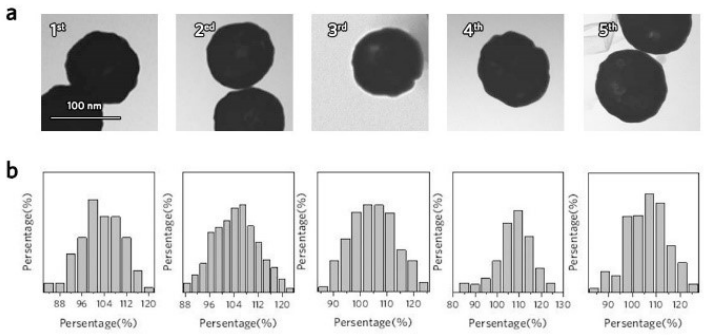

Fig. S7 TEM images (a) and corresponding histograms (b) of AHSNs obtained after each heating and cooling cycle. The average diameters are 105.8, 104.6, 104.8, 108 and 106.2 nm, respectively.

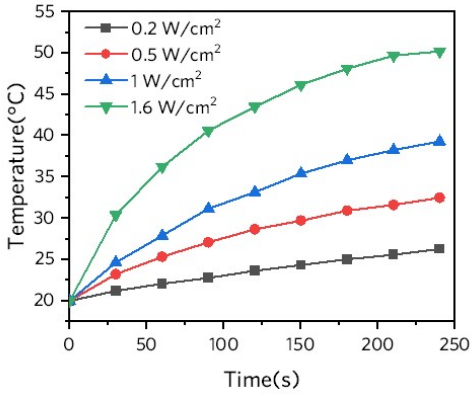

Fig. S8 Photothermal-heating curves of AHSNs (10 μg mL^{-1}) under NIR laser illumination at various laser power densities.

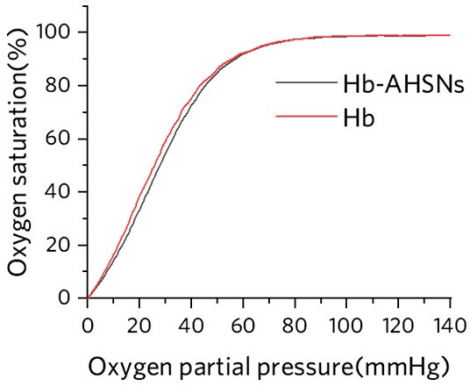

Fig. S9 Oxygen dissociation curves (b) of Hb and Hb-AHSNs.

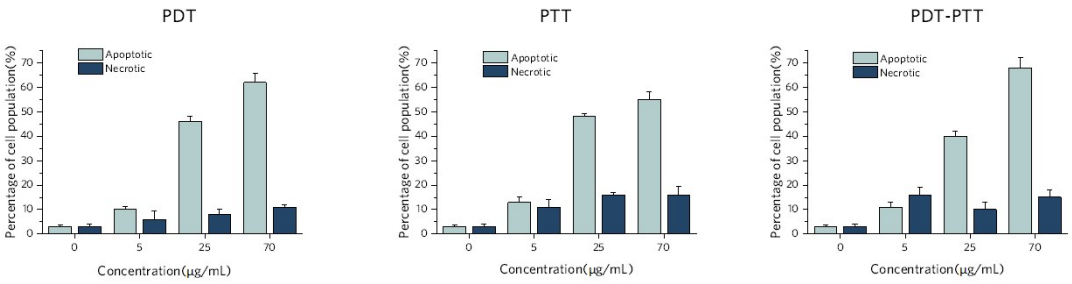

Fig. S10 In SW620 cells, the populations of apoptotic cells and necrotic cells were evaluated as the percentage of total cells for 12 h incubation.

Table S1 The variables and levels used for RSM.

Factors	Level		
	–1	**0**	**1**
NPs concentration （μg/mL）	20	70	120
PTT dose (J/cm^2)	1	6.5	12
PDT dose (J/cm^2)	0.5	2.75	5

Table S2 The studied variables levels in each run with experimental response based on CCD method.

No.	NPs concentration （μg/mL）	PTT dose (J/cm^2)	PDT dose (J/cm^2)	SF (experimental)
1	20	1	0.5	1.99
2	20	1	5	3.24
3	20	6.5	2.75	4.36
4	20	12	0.5	2.43
5	20	12	5	2.55
6	70	1	2.75	3.45
7	70	6.5	0.5	1.46
8	70	6.5	5	3.90
9	70	6.5	2.75	6.30
10	70	12	2.75	2.56
11	120	1	0.5	1.18
12	120	1	5	2.175
13	120	6.5	2.75	3.15
14	120	12	0.5	1.515
15	120	12	5	1.61

Table S3. Variance analysis of the model equation.

Parameter	Coefficient Estimate	F-value	p-value
β_0	0.2457	0.292	0.591
β_1	0.0515	44.212	2.22×10^{-9}
β_2	0.1309	2.5	0.117
β_3	0.9813	13.319	0.0004
β_4	–0.00041	55.675	5.17×10^{-11}
β_5	–0.01145	4.145	0.045
β_6	–0.1096	7.229	0.0085
β_7	–0.00027	0.628	0.43
β_8	0.000208	0.05	0.823
β_9	0.0396	16.842	8.91×10^{-5}

References

1 J. Nam, W.-G. La, S. Hwang, Y. S. Ha, N. Park, N. Won, S. Jung, S. H. Bhang, Y.-J. Ma, Y.-M. Cho, M. Jin, J. Han, J.-Y. Shin, E. K. Wang, S. G. Kim, S.-H. Cho, J. Yoo, B.-S. Kim and S. Kim, *ACS Nano*, 2013, **7**, 3388-3402.