Supplementary Information

Preparation of hypocrellin B nanocages in self-assambled apoferritin for enhanced intracellular uptake and photodynamic activity

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Experimental section

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Entrapment efficiency (EE) and drug loading (DL)

Drug loading (DL) and entrapment efficiency (EE) of HB-AFT NPs were determined by high performance liquid chromatography (HPLC, Agilent 100 series, USA). pH of samples was firstly adjusted to 2.0 by 1M HCl to release HB. After that HB was extracted by acetonitrile for about one hour. The solution was then centrifuged for 10 min at 12000 r/min. 10 μL of supernatant solution was injected at least three times into a C18 column (ACE5, 4.60 mm×250 mm, 5 μm). Mobile phase was made up of acetonitrile (A) and 0.5% acetic acid (B). And the gradient is 0–20 min 50% A–90% A. Flow rate was 1.0 mL/min and the detection wavelength was set at 265 nm. The measurement was performed at least three times.

Dark toxicity

A preliminary study on the dark toxicity of free HB and HB-AFT NPs at various concentrations (2, 4, 8, 16, 32 and 64 μM) for MDA-MB-231 cells was performed. In these experiments, 1×10^4 cells/well were plated in 96-well plates and incubated at 37°C overnight. After removing the old culture medium, cells were incubated with free HB and HB-AFT NPs in the dark for 24 h. Afterwards, 20 μL MTT solution (5 mg/mL) was added into each well and further incubated for 4 h followed by adding 150 μL DMSO to dissolve the resulting formazan crystal. Finally, the absorbance intensity was recorded in a Bio-Tek μQUENT MQX200 spectrophotometric microplate reader (Bio-Tek Instruments, USA) at 570 nm. The relative cell viability (%) was assessed as a percentage relative to the untreated control cells.

Photocytotoxicity assay

Before photodynamic treatment, cells were incubated with free HB and HB-AFT NPs for 4 h in serum-free medium. Cells were then washed with PBS and maintained in fresh medium. Afterwards, cells were irradiated with LED at a wavelength of 630 nm and dose of 106 mW/cm2 for 2 s, 4 s, 8 s, 16 s, 32 s and 64 s, respectively. After irradiation, the cells were further incubated overnight and the cell viability was evaluated by MTT assay.

Cell uptake

MDA-MB-231 cells (1×105 cell/well) were seeded in a 24-well plate. After overnight culture, cells were then incubated with fresh medium containing 2 μM HB-AFT NPs or free HB in the dark for serial durations (1, 2, 4, 6, 8,12 and 24 h). Cells were washed with PBS for three times. Subsequently, cells were lysed with 0.5 mL of 2% sodium dodecyl sulfate (SDS) for 2 h and then treated with 0.1 mL NaOH (1 M) for 1 h at 37°C to make sure the completely soluble of HB.

To better understand the cellular uptake of HB-AFT NPs, cells were incubated with free AFT (500 nM) for 2 h before adding HB and HB-AFT NPs. The lysed cells were centrifuged at 5000 revolutions per min (rpm) for 5 min and fluorescence intensity of the supernatant was measured under 478 nm excitation with a multimode plate reader (DTX-880, Beckman Coulter, CA, USA). The measurements were performed in triplicate.

Overnight-cultured MDA-MB-231 cells (2×104 cells/chamber) were respectively treated with HB-AFT NPs and free HB at a final concentration of 2 μM in a chamber slide

(SPL life science, Korea). After incubation, the cells were imaged by flow cytometry and fluorescence microscopy (Carl Zeiss, Germany) respectively with 488nm wavelength laser for excitation of the HB. Differential interference contrast (DIC) confocal images were collected simultaneously.

Cell localization

For the localization experiments, cells (1×10^4 cells/chamber) cultured overnight were incubated with HB or HB-AFT NPs (2 μM) in the dark for 4 h. The thoroughly washed cells were then stained with 0.5 ml of MitoTracker Green FM (10 nM), LysoTracker Green DND-26 (50 nM), ER-Tracker Green (100 nM) and BODIPY FL C5 ceramide (2 μM) for 30 min, respectively. All trackers were perchursed from Life Technologies Inc, USA. After rinsed with PBS for three times, cells were subsequently fixed with 4% paraformaldehyde for 30 min. After rinsed with PBS, the cells were observed under confocal laser scanning microscope (D-Eclipse C1, Nikon, Japan) at the excitation wavelength of 488 nm. Red and green emission channels were selected respectively for HB and trackers. Sequential rather than simultaneous acquisition was used to avoid bleed-through between the two fluorescent channels. Differential interference contrast (DIC) confocal images were collected simultaneously.

Intracellular ROS generation

Intracellular ROS generation was determined by flow cytometry using 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) staining. DCFH-DA is a non-fluorescent probe. When taken up by cells, it is deacetylated by intracellular esterases forming dichlorodihydrofluorescein (DCFH). DCFH is quickly converted to fluorescent dichlorofluorescein (DCF) in the presence of intracellular ROS. MDA-MB-231 cells were seeded in 35×10 mm dishes at a density of 4×105 cells/dish. Following incubation for 24 h, cells were treated with HB or HB-AFT NPs for 2 h in the dark. Cells were then washed three times with PBS and incubated with 10 μM DCFH-DA at 37°C for 15 min. After thoroughly washed, cells were exposed to light (106 mW/cm2, 16 s) and the fluorescence intensity of DCF were detected by BD FACSCalibur flow cytometer (USA) with the excitation wavelength of 488 nm and signals were acquired at the FL-2 channel. In each analysis, 10,000 cells were assayed.

Estimation of the mitochondrial function

Depolarization of Mitochondrial membrane potential was evaluated by the cationic dye JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3' tetraethyl-benzimidazolylcarbocyanine iodide). 3×10^5 cells were seeded in 35 × 10 mm dishes and incubated overnight. HB or HB-AFT NPs were incubated with cells in the dark for another four hours. The cells were then washed three times and exposed to 630 nm light for 16 s. Sixteen hours after the treatment; cells were harvested and suspended in PBS. 10 μL (200 μM) of JC-1 was added to 1 mL cells suspension incubated for 15 min at 37 °C. Following incubation, the cells were washed with PBS again and were subject to flow cytometer.

Cell nuclear staining

MDA-MB-231 cells (2×105 cells/well) were incubated for 16 h in 35×10 mm glass bottom dishes after the treatment of LED light and HB. The cells were stained with Hoechst 33342 (1 mg/mL, stock solution was 10 mg/mL in sterile water) for 15 min at 37 °C. The cells were washed three times with PBS gently and visualized immediately using a fluorescence microscopy (Carl Zeiss, Germany). Emission was collected at 460 ± 20 nm upon excitation at 405 nm.

Cell apoptosis

Cells were incubated with HB and HB-AFT NPs in the dark for 4 h. Unbound drugs were rinsed away and the cells (except for control groups) were exposed to 630 nm LED light at a dose of 106 mW/cm^2 for 16 s. Before analysis, cells were incubated for 16 h at 37 °C. Finally, apoptosis was measured using flow cytometry with Annexin V-FITC and propidium iodide (PI) staining (Life Technologies Inc, USA). Briefly, cells were harvested and washed with cold PBS for two times. Next, cells from each sample were re-suspended in 500 μL Annexin V-FITC binding buffer containing 5 μL Annexin V-FITC and 5μL PI and incubated at room temperature for 15 min. The samples were analyzed using BD FACSCalibur flow cytometer (USA). The excitation wavelength was 488 nm and signals were acquired in the FL1 (530 nm) and FL2 (585 nm) channels. In each analysis, 10,000 cells were assayed.

Results and discussion

Table S1. Diameters of apoferritin treated by different factors

	pH change	Dialysis	DMSO	HB	Diameter (nm)
1	−	−	−	−	12.8 ± 2.4
2	+	−	−	−	12.7 ± 2.2
3	+	+	−	−	12.5 ± 2.4
4	+	+	+	+	12.9 ± 2.7
5	+	+	+	+	12.7 ± 3.0

Table S2. Diameters of HB-AFT NPs stored at 4°C for different periods

Time	Diameter (nm)
Zero day	12.7 ± 3.0
One week	12.7 ± 2.9
One month	13.0 ± 4.7
Three months	23.7 ± 7.0

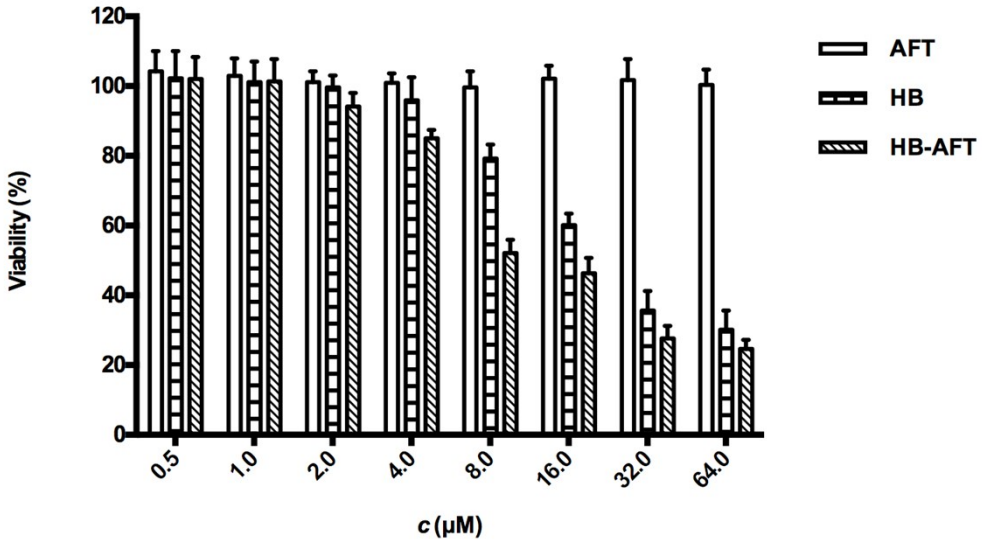

Fig. S1 Dark toxicity (%) of AFT, HB, and HB-AFT NPs on MDA-MB-231cells measured under different concentrations assessed by MTT assay. The *c* represents the concentration (in μM) of HB.

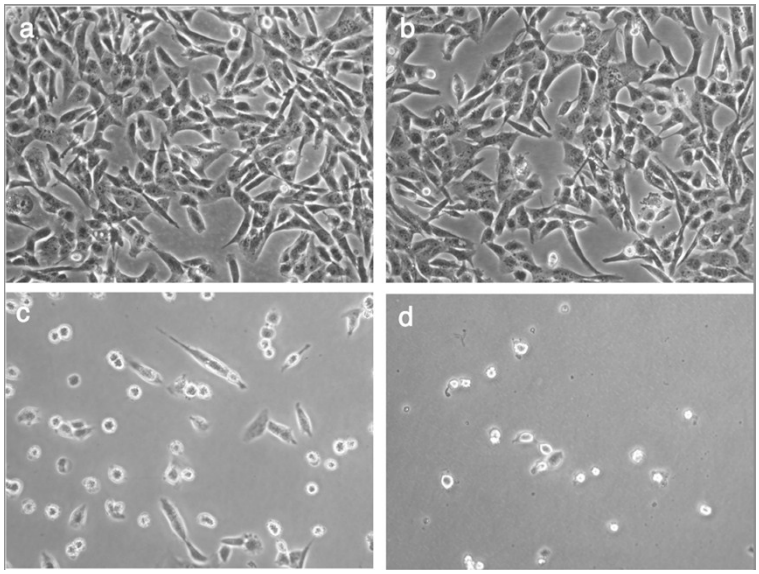

Fig. S2 MDA-MB-231 cells were observed by an inverted microscopy (200×). (a) Sham control, (b) apoferritin with 8s 630 nm light irradiation, (c) HB with 8s 630 nm light irradiation, (d) HB-AFT NPs with 8s 630 nm light irradiation.

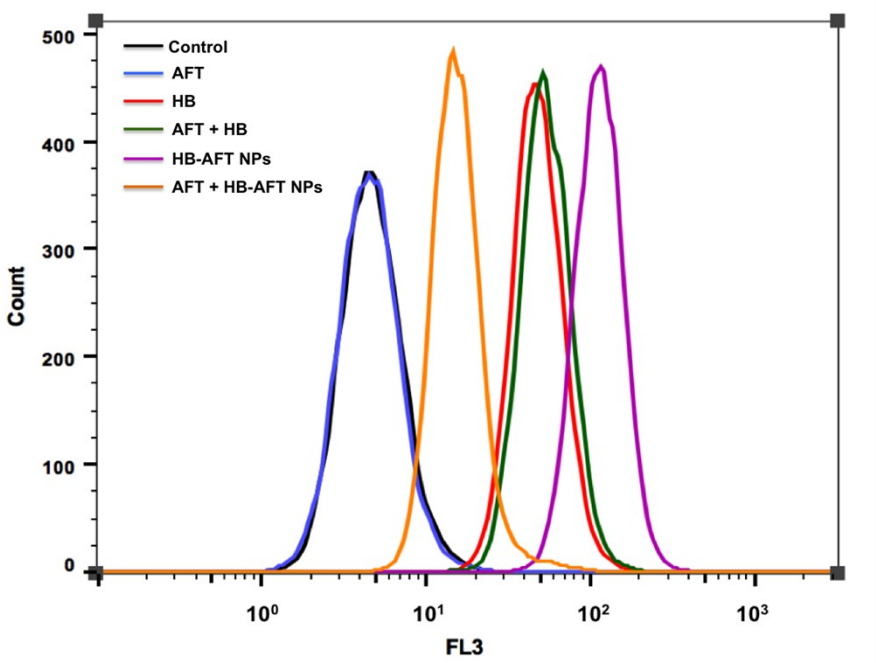

Fig. S3 Cell uptake of HB and HB-AFT NPs detected by flow cytometry in the presence and in the absence of AFT. The concentration of HB was 2μM, the amount of HB in HB-AFT NPs was equivalent to that of free HB.

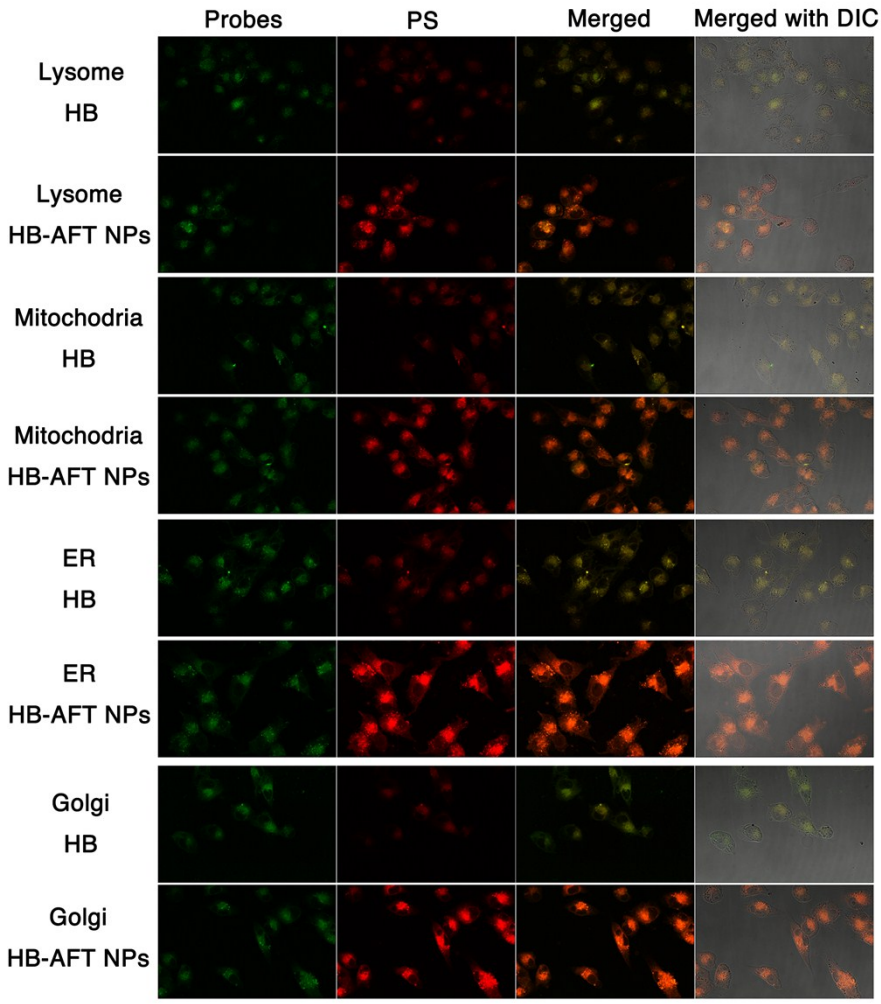

Fig. S4 Cellular localization of HB and HB-AFT NPs in MDA-MB-231 cells after 4 h incubation. Green color: probe of organelles, Red color: intracellular HB.

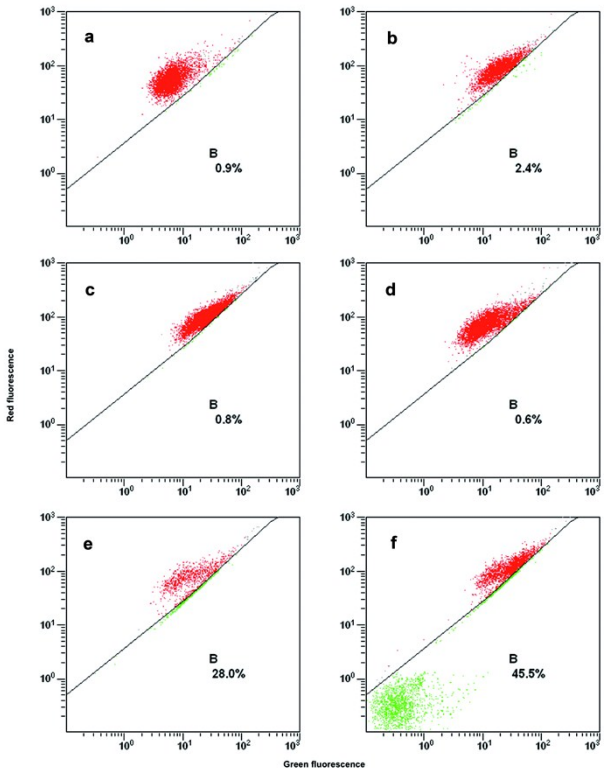

Fig. S5 The MMP of the cells 16 hours after treatment：(a) sham control, (b) light alone, (c) HB alone, (d) HB-AFT NPs alone, (e) light activated HB, (f) light activated HB-AFT NPs.

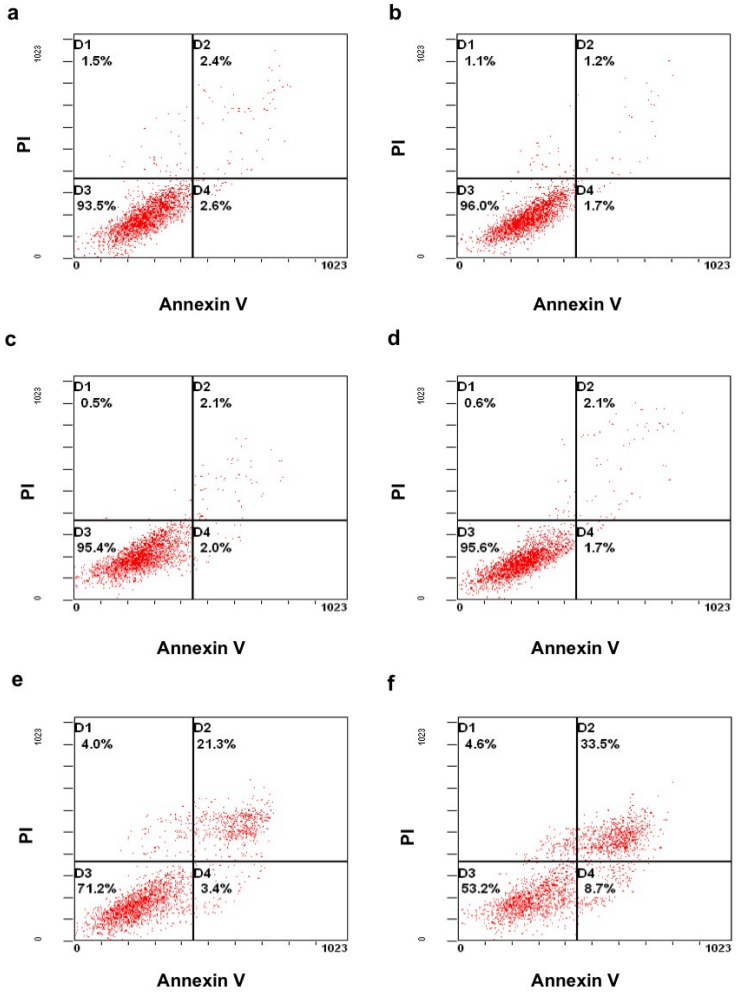

Fig. S6 Apoptosis of MDA-MB-231 cells after treated with LED light with the energy density of 1.70 J/cm^2: (a) sham control, (b) light alone, (c) HB alone, (d) HB-AFT NPs alone, (e) light activated HB, (f) light activated HB-AFT NPs.