

Supporting Information for

**Folic acid conjugated titania-silica porous hollow nanosphere for
improved topical photodynamic therapy**

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MATERIALS & METHODS

Fabrication of FA-HNS-PpIX: Silica nanospheres were synthesized according to the Stöber method. First, 2.9 mL of tetraethyl orthosilicate (TEOS; Aldrich, St. Louis, MO) were mixed with ethanol (79 mL), ammonium hydroxide solution (3.9 mL), and water (1.4 mL). The mixture was stirred in a vessel for 12 h under 40 °C temperature for synthesizing silica nanospheres. Subsequently, 3 mL of titanium (IV) isopropoxide (TTIP; Aldrich) was added into the above mentioned solution. The solution was stirred for 12 h at room temperature. The resultant solution was added into 12 mL of ammonium hydroxide solution and sonicated for 12 h. In order to load the protoporphyrinIX (PpIX; Aldrich) into the dispersed HNS solution, the PpIX was dissolved in DMSO at a concentration of 10 mg mL⁻¹ and 2 mg of HNSs were added. The mixture was stirred overnight, and then dried under vacuum. The γ -glycidoxy propyl trimethoxysilane (GPTS, 1 mL) were introduced into ethanol solution with HNS-PpIX for overnight. Following, 100 mg of folic acid (FA; Aldrich) was added and stirred overnight. The solution was thoroughly washed to remove unreacted FA.

Characterization: Transmission electron microscopy (TEM) images were obtained with a JEOL EM-2000 EX II microscope. The surface charge (zeta potential) and hydronamic size distribution were measured by ELS-8000 (Otsuka Electronics, Japan) and DLS-7000 (Otsuka Electronics, Japan). The Brunauer-Emmett-Teller (BET) surface area, average pore volume and Barrett-Joyner-Halenda (BJH) pore distribution was analyzed by ASAP 2000 (Micromeritics, USA). Fourier transform infrared (FTIR) spectra were obtained using a FT-IR Frontier (PerkinElmer, USA) in absorption mode at a resolution of 64 cm⁻¹. Ultraviolet-visible (UV-Vis) spectra were acquired using a Lambda-20 spectrometer (PerkinElmer, USA). Photoluminescent absorption spectra were gained with JASCO FP-6500 spectrofluorometer.

PpIX release profile: The synthesized FA-HNS-PpIX was diluted with phosphate buffered saline (PBS) (0.3 mg mL⁻¹ of FA-HNS-PpIX). PpIX was released from FA-HNS-PpIX and detected by UV-vis spectrometry with 3 min of intervals. In each experiment, the FA-HNS-PpIX was centrifuged for separating released PpIX from FA-HNS-PpIX. After the centrifugation, the supernatant was collected and the relative PpIX release % was calculated. The cumulative PpIX

release % was calculated by **Equation (1)**. Where m_{total} is the total mass of PpIX and $m_{released}$ is a mass of released PpIX from FA-HNS-PpIX.

$$PpIX_{release}(\%) = \frac{m_{released}}{m_{total}} \times 100 \quad (1)$$

PpIX loading efficiency: After loading PpIX in HNSs and centrifugation, the supernatant was collected and the residual PpIX amount was measured by UV-vis spectrometry. The PpIX loading capacity was calculated by **Equation (2)**. Where m_{total} is the total mass of PpIX and $m_{residual}$ is a mass of residual PpIX in the supernatant. $m_{HNS-PpIX}$ is mass of PpIX loaded into HNSs. The 1.645 μM is the PpIX encapsulated in HNS-PpIX (PpIX encapsulating efficiency: 92.12 %). The excellent hydrophobic PpIX encapsulating ability of HNS is proved. Furthermore, after folic acid treatment, the PpIX loading efficiency is calculated as 27.31 % by **Equation (3)**. This result showed about 4.5 times higher hydrophobic porphyrin loading capacity by comparing with other 212 nm sized peptide micelle structure ((C18)2K]2KR8GRGDS; porphyrin loading efficiency: 6 %).¹

$$PpIX_{encapsulating}(\%) = \frac{m_{HNS-PpIX}}{m_{total} - m_{residual}} \times 100 \quad (2)$$

$$PpIX_{loading}(\%) = \frac{m_{PpIX}}{m_{FA-HNS-PpIX}} \times 100 \quad (3)$$

Detection of 1O_2 generation: In the absence of the cells, singlet oxygen (1O_2) generated by PpIX were detected by 9,10-anthracenediyl-bis(methylene) dimalonic acid (ABDA; Aldrich). ABDA can react with 1O_2 and causes a decrease of ABDA absorption peakd at 380 nm. The solution of FA-HNS-PpIX were prepared by dispersing in 0.1 M phosphate buffered saline (PBS). ABDA (70 $\mu g mL^{-1}$) and equivalent 20 $\mu g mL^{-1}$ of PpIX solutions were mixed and vortexed for dispersion. Then, the mixture was irradiated with visible light. As a light source, 5.6 mW cm^{-2} LED light (ING, China) was used. The irradiation experiment was performed at room

temperature. The generation of $^1\text{O}_2$ was monitored by recording the decrease in ABDA absorption peak at different irradiation time scale.

Cell culture: Human breast cancer MCF-7 and SK-BR-3 cell lines were purchased from ATCC (American type culture collection). They were cultured with RPMI-1640 medium with 10% fetal bovine serum, and 1% penicillin-streptomycin solution in a 5% CO_2 incubator at 37 °C. Cells were placed in 75T flask and maintained between 1×10^5 and 1×10^6 cells mL^{-1} .

ATP luminescent assay: The viability of the FA-HNS-PpIX treated cells were measured using Cell-Titer® glow lumiscent cell viability assay (Promega, Madison, WI, USA). The cell viability was calculated by counting the amount of ATP in metabolically active cells. The lumiscent signal was generated when the beetle luciferin is transformed into oxyluciferin by a recombinant luciferase in the presence of ATP. The cells were seeded at a density of 5000 cells per well in white luminescent 96-well plates (SPL, USA) and treated with different concentrations of the FA-HNS-PpIX nanoparticles ($10 \mu\text{g mL}^{-1}$, $20 \mu\text{g mL}^{-1}$, $30 \mu\text{g mL}^{-1}$, $40 \mu\text{g mL}^{-1}$, $50 \mu\text{g mL}^{-1}$, and $60 \mu\text{g mL}^{-1}$) for 24 h. The plates were washed for 3 times for remove residual FA-HNS-PpIX, and irradiated for different time scale (0 min, 5 min, 10 min, 15 min). After this process, the supernatant was removed and the following procedure were carried out as supplier's instructions. The intercellular ATP content was detected by Victor³ Multilabel Readers (Perkin Elmer, USA).

Cellular uptake of FA-HNS-PpIX: The cellular uptake of HNSs and FA-HNS-PpIX in MCF-7 and SK-BR-3 cells were observed by using transmission electron microscopy (TEM). Both cells were seeded in culture dishes (Nunc, Thermo Fisher Scientific, USA) and cultivated for 24 h. After incubation of HNSs and FA-HNS-PpIX (25 mg mL^{-1}) for 24 h, cells were fixed by using Karnovsky's fixative (mixed with 2% paraformaldehyde and 2% glutaraldehyde) for 2 h at 4 °C. Samples were washed with distilled water and post-fixation was conducted by using 1% osmium tetroxide at 4 °C for 2 h. The samples were washed with 0.1 M PBS and stained with 0.5% uranyl acetate. Then, ethanol and propylene oxide were used for dehydration of the samples. Cells were embedded in Spurr's resin and sliced by ultramicrotome. The samples were observed by TEM (JEM1010, JEOL) at 80 kV.

Amount of intercellular PpIX: To quantify amount of the intercellular PpIX, MCF-7 and SK-BR-3 cells were plated in sterile culture dishes (Nunc, Thermo Fisher Scientific, USA) at a density of 5×10^5 per dish and incubated for 24 h. The cells were respectively incubated with HNS, PpIX, FA-HNS, HNS-PpIX, and FA-HNS-PpIX for 24 h. The particles were concentrated with equivalent PpIX concentration (equivalent $13.8 \mu\text{g mL}^{-1}$ of PpIX). Then, plates were washed with 0.1 M PBS for thrice times and PpIX internalized cells were detached with 0.4 % trypsin-EDTA. The detached cells were collected by centrifuge for 120 rpm and analyzed by flow cytometry (FACS Calibur, BD Bioscience, USA).

Live cell observation of cell death: The MCF-7 and SK-BR-3 were cultured at a density of 3×10^4 cells per well in cover slip placed 12-well plates (Nunc, Thermo Fisher Scientific, USA) and incubated for 24 h. Then, cells were treated with FA-HNS-PpIX (10, 20, 30, 40, 50, and $60 \mu\text{g mL}^{-1}$) for 24 h. After FA-HNS-PpIX incubation, the plates were washed with 0.1 M PBS for 3 times. Subsequently, the cells were irradiated with different time scale (0, 5, 10, and 15 min). Complete the irradiation step, DeadEnd™ Fluorometric TdT-mediated dUTP Nick-End Labeling (DeadEnd™ Fluorometric TUNEL assay; Promega, USA) and Fluoroshield™ with 4',6-Diamidino-2-phenylindole dihydrochloride (Fluoroshield™ with DAPI; Aldrich) staining was carried out. Live cell fluorescent images were obtained by using a Delta Vision RT imaging system (Applied Precision, Issaquah, WA) and Cascade II electron-multiplying charge-coupled device (EMCCD) camera. The dead cells were distinguished by using a fluorescence microscope with appropriate filters. The dead cells exhibited bright green fluorescence at 488 nm, while viable cells emitted weak fluorescence.

Cell viability test: The viability of cells treated with FA-HNS-PpIX was measured by using dye exclusion method. This vital stain method used a 0.4% trypan blue solution (Aldrich). The viable cells were not stained by trypan blue solution. On the other hand, non-viable cells were stained by this solution and classified as dead cells. To perform the assay, cells were cultivated in cover slip placed 12-well plates (Nunc, Thermo Fisher Scientific, USA) at a density of 7×10^3 cells per well and incubated for 24 h. Then, cells were treated with FA-HNS-PpIX (10, 20, 30, 40, 50, and $60 \mu\text{g mL}^{-1}$) for 24 h. In order to remove the unbound FA-HNS-PpIX, culture

medium was removed and cells were washed with 0.1 M Hank's Balanced Salt Solution (HBSS). Subsequently, cells were irradiated with different time scale (0, 5, 10, and 15 min). For control experiment, both cell lines were incubated with different particles (HNSs, FA-HNS and HNS-PpIX were treated at the equivalent 5 $\mu\text{g mL}^{-1}$ of HNSs concentration; 5 $\mu\text{g mL}^{-1}$ of HNSs is a carrier concentration in a 60 $\mu\text{g mL}^{-1}$ of FA-HNS-PpIX concentration). The irradiated cells were stained with trypan blue solution and observed by optical microscope (Eclipse E600, Nikon). Cell viability was determined as the percentage of the unstained cells over the untreated cell control.

Intercellular H_2O_2 production: To determine the PDT induced cell damage, intercellular hydrogen peroxide (H_2O_2) production was measured. The cells were cultivated in 96-well plate (SPL; USA) at a density of 5000 cells per well, and different concentrations of FA-HNS-PpIX were incubated with the cells for 24 h. Then, each well was irradiated with different time scale (0 min, 5 min, 10 min, and 15 min) and stained with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). The H_2DCFDA specifically detect intercellular H_2O_2 , which is precursor and intermediate of interbody reactive oxygen species (ROS). After reacted with H_2O_2 , H_2DCFDA was converted to H_2DCFDA , exhibiting green fluorescence. The intercellular H_2O_2 was detected by Victor³ Multilabel Readers (Perkin Elmer, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Alteration of mitochondrial membrane potential: To investigate the change of the mitochondrial membrane potential, cells were seeded in 8-well Lab-Tek II chambered cover glass (Nunc, Thermo Fisher Scientific, USA) at a density of 4000 cells per well and treated with FA-HNS-PpIX (60 $\mu\text{g mL}^{-1}$). After incubation, the cells were irradiated with LED light (0 min, 5 min, 10 min, and 15 min) and stained with 10 μM Rhodamine-123 (Rh-123; Invitrogen, Grand Island, NY). Rh-123 is a specific dye that can directly act on mitochondria. Delta Vision RT imaging system (Applied Precision, Issaquah, WA) equipped Cascade II electron-multiplying charge-coupled device (EMCCD) camera was used to get a live cell fluorescent image. The Rh-123 dye was excited by a wavelength of 505 nm and emitted 560 nm green fluorescence.

Quantification of mitochondrial dysfunction by flow cytometry: Mitochondrial dysfunction in cells was observed by Rh-123 staining followed by flow cytometric measurement of the fluorescence. Cells were incubated in sterile culture dishes (Nunc, Thermo Fisher Scientific, USA) at a density of 5×10^5 per dish and incubated for 24 h. Then, cells were treated with FA-HNS-PpIX (10, 20, 30, 40, 50, and $60 \mu\text{g mL}^{-1}$) for 24 h. After washing with 0.1 M PBS, cells were suspended in 0.1 M HBSS, stained with Rh-123, and analyzed by flow cytometry (FACs Aria, BD Bioscience, USA).

FT-IR spectroscopy of HNS, HNS-PpIX, and FA-HNS-PpIX

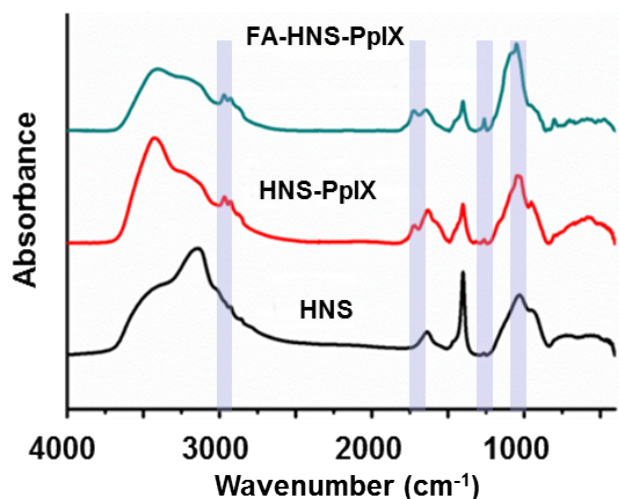


Fig. S1 Infrared spectroscopy absorbance spectra of HNS, FA-HNS, and FA-HNS-PpIX.

Fourier transform infrared spectroscopy (FT-IR) confirmed the each step of synthesis (Fig. S1 and Table S1). The spectrum of the HNS had characteristic peaks at 952, 1075, and 1402 cm⁻¹ corresponding to Si–O–Ti vibrations, Si–O stretching, and Si–O stretching overtone vibrations, respectively.² HNS–PpIX had a C–N stretching band at 1076 cm⁻¹, a C=O stretching band at 1724 cm⁻¹, and a C=C stretching vibration at 2949 cm⁻¹, indicating the presence of PpIX within the HNS cavity.³ The increased intensities of the C–N stretching band at 1076 cm⁻¹ and the C=O stretching band at 1724 cm⁻¹ confirmed the surface treatment with FA.⁴ The characteristic peaks of FA–HNS–PpIX at 698 cm⁻¹ (CH₂ rocking vibration of Si–CH₂–R) and 1261 cm⁻¹ (aromatic C–H vibration) demonstrated the successful surface functionalization of FA.⁵ The peak assignments were summarized in Table S1.

Table S1. FT-IR assignment of materials.

Materials	Wavenumber (cm ⁻¹)	Assignments
HNS	3239	-OH stretching
	1634	-OH vibration
	1402	Si-O overtone
	1025	Si-O stretching
	952	Si-O-Ti
PpIX	2949	C=C symmetric stretching
	1724	C=O stretching
	1076	C-N stretching
FA	1724	C=O stretching
	1261	aromatic C-H vibration
	1076	C-N stretching
	698	Si-CH ₂ -R vibration

Surface modification of the FA-HNS

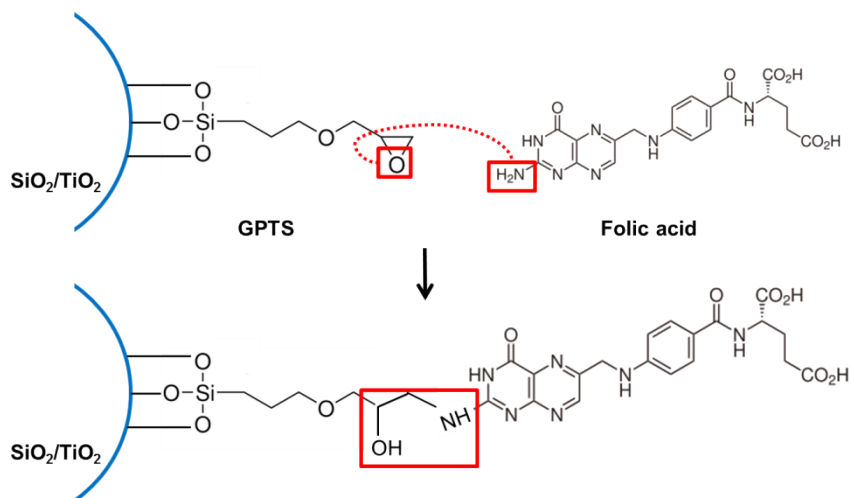


Fig. S2 Schematic illustration of reaction between GPTS and folic acid

In this manuscript, γ -glycidyloxy propyl trimethoxysilane (GPTS) was used for folic acid conjugation on the surface of the HNS. The GPTS could form covalent bond with amine group of folic acid (Fig. S2).⁶ In previous research, carboxylic group of the folic acid plays a key role in high binding affinity between folic acid and folate receptor.⁷ In this regard, GPTS is an appropriate silane coupling agent for folic acid functionalization without any disturbance on its affinity with folate receptor. By using GPTS, a folic acid was successfully introduced on the surface of the HNS *via* covalent bond.

DLS size distribution

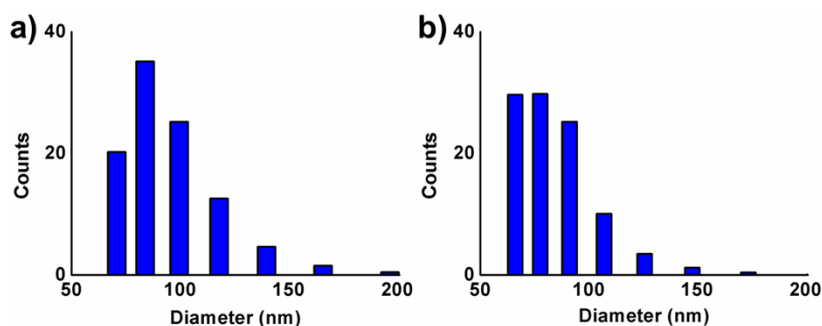


Fig. S3 Hydronamic diameter of HNS and FA-HNS determined by the DLS

The dynamic light scattering (DLS) data confirmed that the HNSs had an average diameter of 83.6 ± 18.4 nm with a narrow size distribution (Fig. S4a). After FA treatment, the diameter of the HNSs increased by 10 nm, while the dispersity and uniformity in size and shape were maintained (Fig. S4b). The particle size change was caused by the FA conjugation to the surface of the HNS. The diameter difference between TEM and DLS was contributed by the hydration sphere and outer FA layer.⁸

Nitrogen adsorption isotherm and pore size distribution of HNSs

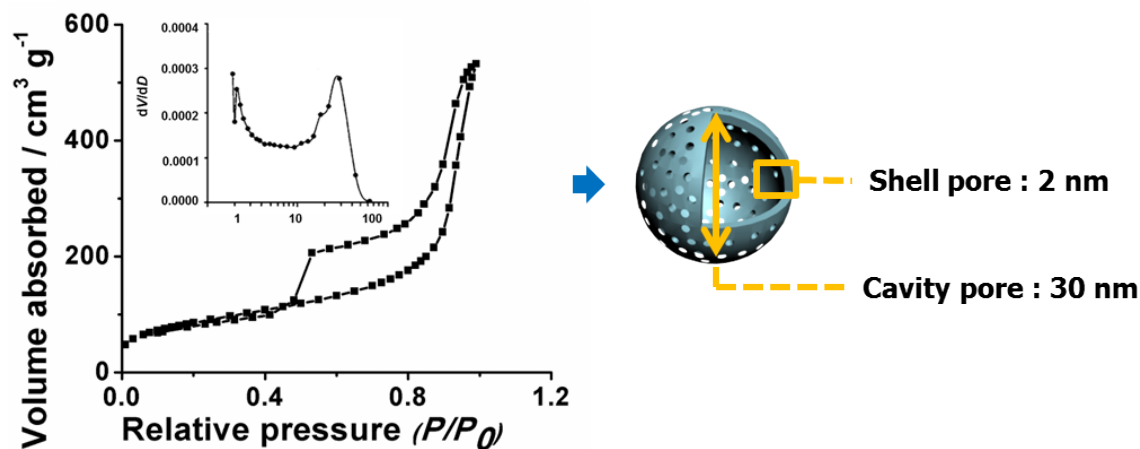


Fig. S4 Nitrogen adsorption isotherm of the HNSs; pore volum is $0.92 \text{ cm}^3 \text{ g}^{-1}$, BET surface area is $313 \text{ m}^2 \text{ g}^{-1}$. Inset: The nitrogen adsorption isotherm exhibits the pore size distribution of HNSs (cavity size: 30 nm, inter-shell pore: 2 nm).

Fig. S4 showed the nitrogen adsorption isotherm of HNSs. HNSs had a Brunauer-Emmett-Teller (BET) surface area of $313 \text{ m}^2 \text{ g}^{-1}$ and an average pore volume of $0.92 \text{ cm}^3 \text{ g}^{-1}$. The Barrett-Joyner-Halenda (BJH) pore distribution confirmed the 30 nm cavity size and 2 nm inter-shell pore of HNSs. This data indicates a mesoporous structure of synthesized HNSs. Furthermore, the $^1\text{O}_2$ generated by PDT could pass through 2 nm inter-shell pore of HNSs, directly stimulated the cancer cells, and finally led destruction of cancer cell. In this point of view, HNSs are suitable carrier agent in PDT for cancer cells.

Solvent dependent behavior of PpIX in FA-HNS-PpIX

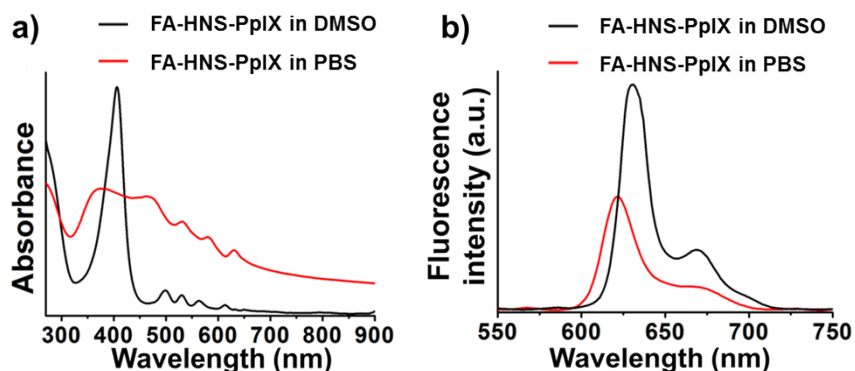


Fig. S5 a) UV-visible spectroscopy absorption spectra and b) fluorescence intensity of FA-HNS-PpIX in different solvents.

The UV-vis absorption spectra of FA-HNS-PpIX dispersed in different solvents were shown in Fig. S5. In order to compare the behavior of the FA-HNS-PpIX in different solvents, dimethyl sulfoxide (DMSO) and phosphate buffer saline (PBS) were selected. In DMSO, PpIX loaded in FA-HNS-PpIX dissolved as a monomer with a sharp Soret band at 402 nm. PpIX stayed as aggregates in PBS with broadened split Soret band of moderate intensity (Fig. S5a).⁹ Fig. S5b indicated PpIX showing bright fluorescence at 632 nm (λ_{ex} : 402 nm) in DMSO, while aggregated PpIX in aqueous environment exhibited the decrease of fluorescence intensity with a blue shift of emission peak at 620 nm (λ_{ex} : 402 nm). PpIX was fully dissolved in DMSO and came out of the HNS due to hydrophobicity. Contrary to DMSO, PpIX was not soluble in PBS and maintained its aggregation inside of the HNS. This result could be concluded that PpIX would be delivered to tumor cell effectively without any leakage from FA-HNS to vessel. The potential of FA-HNS as PpIX encapsulating and delivery agent was verified in this study.

UV/visible light absorption of HNSs

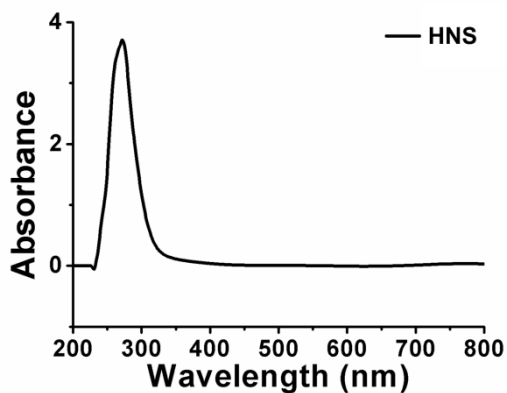


Fig. S6 UV/visible absorption spectrum of HNSs in PBS

The HNSs did not absorb visible light over 400–800 nm (in Fig. S6). An LED light penetrated into the HNSs and reacted with the PpIX. The LED has superior properties to other types of light sources such as intact of healthy cells during irradiation and easy to access in daily life. Therefore, PDT using LED as light source is expected to be effective and have less side effects.

Singlet oxygen ($^1\text{O}_2$) generation of FA-HNS-PpIX

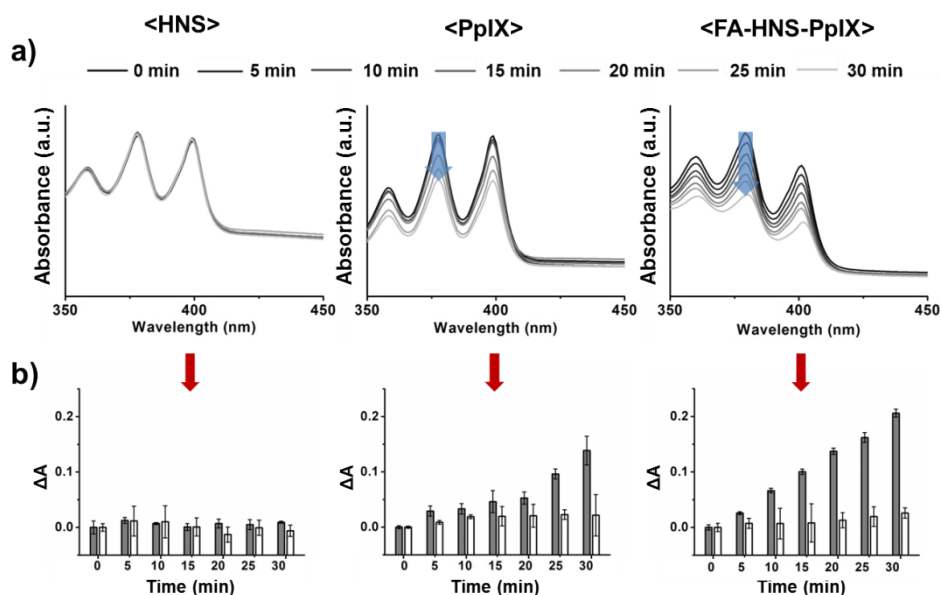


Fig. S7 Photobleaching of ABDA by HNS, PpIX, and FA-HNS-PpIX dispersed in 0.1 M PBS buffer. a) Absorbance spectra of HNS, PpIX, and FA-HNS-PpIX irradiated with different time scale by using visible light. b) The change in absorption intensity of ABDA of HNS, PpIX, and FA-HNS-PpIX (grey: with irradiation, white: without irradiation). Values exhibit mean $\pm$ SD, and each experiment was performed in triplicate.

To confirm the photodynamic activity of the FA-HNS-PpIX, 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) was used for detecting $^1\text{O}_2$.¹⁰ ABDA reacted with the $^1\text{O}_2$ and showed a decrease in the ABDA absorption peak at 380 nm.¹¹ To monitor the amount of $^1\text{O}_2$ generation from the HNS, PpIX, and FA-HNS-PpIX as a function of the irradiation time, the ABDA was added into each solution and irradiated for different times at intervals of 5 min; control samples were placed in a dark room. The calculated ΔA from the PpIX and FA-HNS-PpIX showed noticeable increase in 30 min of irradiation, while the HNS had no effect on the ΔA under both with and without irradiation condition (Fig. S7). The 30 min irradiated FA-HNS-PpIX exhibits 33.78 % larger ΔA value than the ΔA of 30 min irradiated PpIX, implying more $^1\text{O}_2$ is generated in FA-HNS-PpIX. Increased ΔA was induced by the enhanced FA-HNS-PpIX dispersion. Folic acid on the surface of the FA-HNS-PpIX improved particle dispersion in 0.1M

PBS, therefore generated singlet oxygen could diffuse out more in the FA-HNS-PpIX than in the pristine PpIX. Furthermore, the increased FA-HNS-PpIX dispersion gave broader surface area for ABDA oxidation, which induced larger ΔA value in FA-HNS-PpIX.¹² Collectively, FA-HNS-PpIX could generate $^1\text{O}_2$ successfully under irradiation, which is suitable for PDT *in vitro*.

Amount of the APT of the MCF-7 and SK-BR-3 cells.

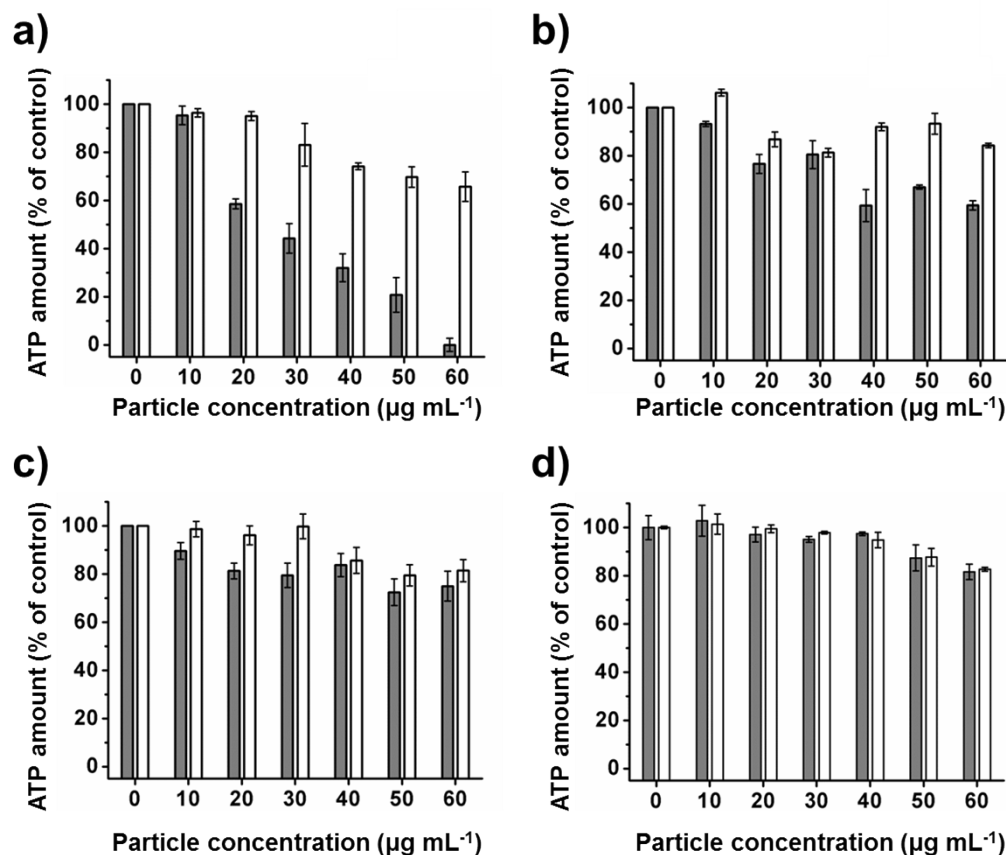


Fig. S8 ATP amount of the MCF-7 and SK-BR-3 cells after being incubated different concentration of the particles for 24 h. The cells were exposed to with and without irradiation condition. MCF-7 cells were incubated with a) FA-HNS-PpIX, and b) PpIX. SK-BR-3 cells were incubated with c) FA-HNS-PpIX, and d) PpIX (grey: 15 min of irradiation, white: without irradiation).

By ATP luminescent assay, the intercellular ATP content was measured by comparing the intensity changes at 560 nm and the ATP related cell viability was calculated and presented in Fig. S8. There was no fluorescence disturbance for FA-HNP-PpIX (emission: 600 nm- 700 nm with ATP luminescent dye). Many PpIX based systems used this method for measuring cell viability.¹³ The ATP based cell viability showed similar result when compared with the dye exclusion based cell viability. The ATP based cell viability showed similar result when compared with the dye exclusion based cell viability. Therefore, it could be concluded that both

types of tested cell viability are trustworthy and reliable than other types of cell viability test.

TEM images of particle-internalized MCF-7 and SK-BR-3 cells

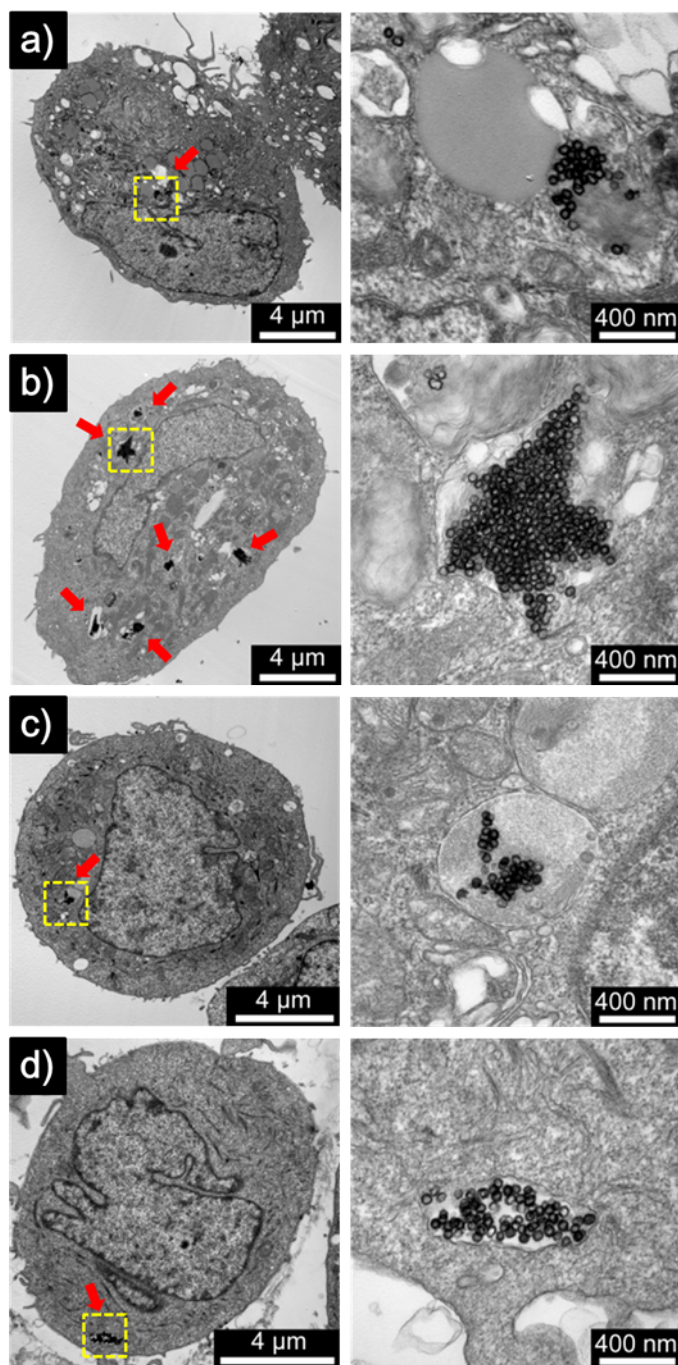


Fig. S9 TEM images of particle-internalized MCF-7 cells and SK-BR-3 cells incubated with HNS and FA-HNS-PpIX for 24 h. Left: TEM images of HNS and FA-HNS-PpIX show cellular uptake in MCF-7 and SK-BR-3 cells. Right: Magnified regions bounded by boxes in the TEM

images, respectively. a) MCF-7 cell with HNS, b) MCF-7 cell with FA-HNS-PpIX, c) SK-BR-3 cell with HNS and d) SK-BR-3 cell with FA-HNS-PpIX (MCF-7 and SK-BR-3 cells were incubated at the equivalent $20\text{ }\mu\text{g mL}^{-1}$ HNS concentration). Red arrows indicate the location of HNS and FA-HNS-PpIX.

To confirm the targeting effect of FA-HNS-PpIX, the intracellular HNSs and FA-HNS-PpIX into SK-BR-3 and MCF-7 were observed by TEM images. Fig. S9 showed both particles were internalized into each cell. The intracellular nanospheres maintained their original shape, and most of the particles were located inside the endosome. Internalization of the particles into the cells did not produce serious cell damage and cell toxicity. A similar amount of HNS internalization into SK-BR-3 and MCF-7 cells exhibited in Fig. S9a and S9c. On the contrary, Fig. S9b and S9d showed that the FA-HNS-PpIX was more internalized in MCF-7 than SK-BR-3 owing to FA-mediated targeting. It was demonstrated that FA functionalized HNSs had targeting ability to specific tumor cell and was suitable as PpIX delivery carrier.

Quantification of PpIX internalization

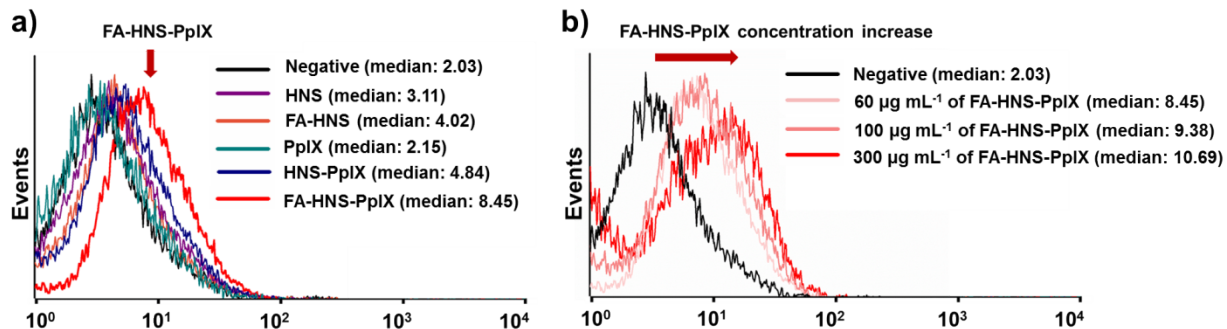


Fig. S10 Flow cytometric analyses of MCF-7 cells with incubation of a) HNS, FA-HNS, PpIX, HNS-PpIX, and FA-HNS-PpIX. B) Flow cytometric analysis of MCF-7 cells with 60, 100, and 300 $\mu\text{g mL}^{-1}$ of FA-HNS-PpIX incubation for 24 h.

A flow cytometry analysis was performed to quantify the intracellular PpIX. Intracellular PpIX was quantified under various carrier conditions using flow cytometry (Fig. S10a; equivalent 13.8 $\mu\text{g mL}^{-1}$ of PpIX treated for MCF-7 cells). When the cells were treated with HNS-PpIX, the fluorescence of the HNS-PpIX increased by 1.98 times compared to that of the pristine PpIX. After FA modification, the fluorescence was significantly shifted by 2 times (from 4.84 to 8.45). Consequently, the FA-HNS-PpIX led to 3.93 times higher fluorescence intensity compared to the pristine PpIX. Furthermore, as FA-HNS-PpIX concentration increased from 60 $\mu\text{g mL}^{-1}$ to 300 $\mu\text{g mL}^{-1}$, the median value also increased from 8.45 to 10.69 (Fig S10b). These results indicated that FA conjugated HNS-PpIX possessed targeting ability for MCF-7 cells and delivery ability for large amount of hydrophobic PpIX into cells. Therefore, HNS could act as an efficient PpIX carrier by folic acid functionalization.

Live cell observation of FA-HNS-PpIX treated MCF-7 and SK-BR-3 cell under irradiation

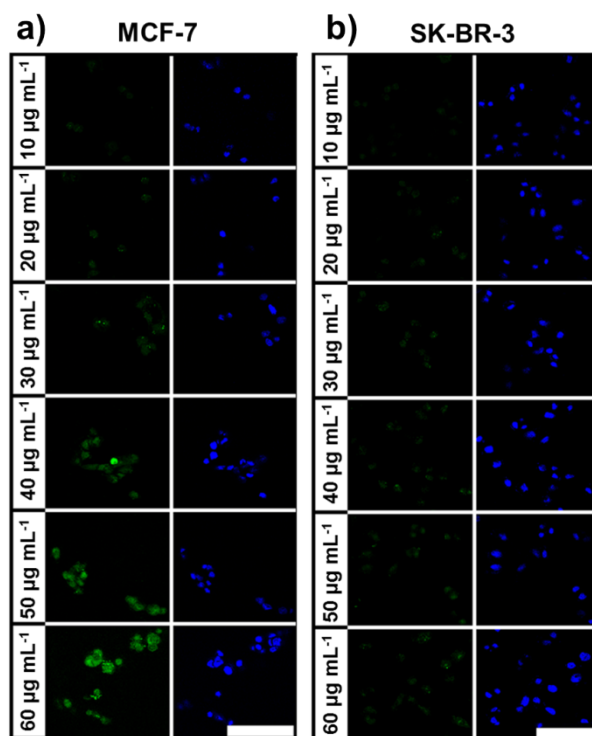


Fig. S11 Livecell fluorescence images of FA-HNS-PpIX treated a) MCF-7 and b) SK-BR-3 cell. From up to the bottom, the concentration of FA-HNS-PpIX is 10, 20, 30, 40, 50 and 60 $\mu\text{g mL}^{-1}$ with 15 min of irradiation. Cells were stained with DeadEnd™ Fluorometric TdT-mediated dUTP Nick-End Labeling (DeadEnd™ Fluorometric TUNEL) assay and Fluoroshield™ with 4',6-Diamidino-2-phenylindole dihydrochloride (Fluoroshield™ with DAPI) for visualizing viable and dead cells. Each fluorescent images were taken at the same time (scale bars: 100 μm).

Live cell observation of FA-HNS-PpIX treated MCF-7 and SK-BR-3 cell under irradiation

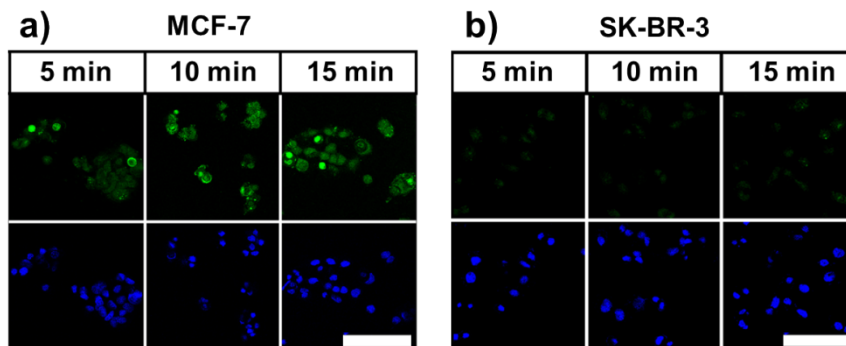


Fig. S12 Livecell fluorescence images of FA-HNS-PpIX treated a) MCF-7 and b) SK-BR-3 cell. From left to the right, the irradiation time is 5 min, 10 min, and 15 min with $60 \mu\text{g mL}^{-1}$ of FA-HNS-PpIX. Cells were stained with DeadEnd™ Fluorometric TdT-mediated dUTP Nick-End Labeling (DeadEnd™ Fluorometric TUNEL) assay and Fluoroshield™ with 4',6-Diamidino-2-phenylindole dihydrochloride (Fluoroshield™ with DAPI) for visualizing viable and dead cells. Each fluorescent images were taken at the same time (scale bars: 100 μm).

To investigate the PDT effect of FA-HNS-PpIX for cancer cell destruction, FA-HNS-PpIX induced phototoxicity was probed by livecell observation. The MCF-7 and SK-BR-3 cells were incubated with different concentration of FA-HNS-PpIX and irradiated with different time scale. After FA-HNS-PpIX treatment and irradiation, the dead cells were stained with DeadEnd™ Fluorometric TdT-mediated dUTP Nick-End Labeling (DeadEnd™ Fluorometric TUNEL). DeadEnd™ Fluorometric TUNEL system could stain dead cells specifically and exhibited green fluorescence.¹⁴ After staining with DeadEnd™ Fluorometric TUNEL system, the samples were treated with Fluoroshield™ with 4',6-Diamidino-2-phenylindole dihydrochloride (Fluoroshield™ with DAPI; blue fluorescence) for visualizing cell nucleus. Fig. S11 showed that brighter green fluorescence was observed in MCF-7 cells with increasing concentration of FA-HNS-PpIX and longer irradiation time. While in Fig. S12, no noticeable cell death was observed for FR- SK-BR-3 cells. These results demonstrated that FA-HNS-PpIX had a highly active targeting ability for FR+ overexpressed cancer cells and excellent PDT effect.

Control experiments on both MCF-7 and SK-BR-3 cell lines

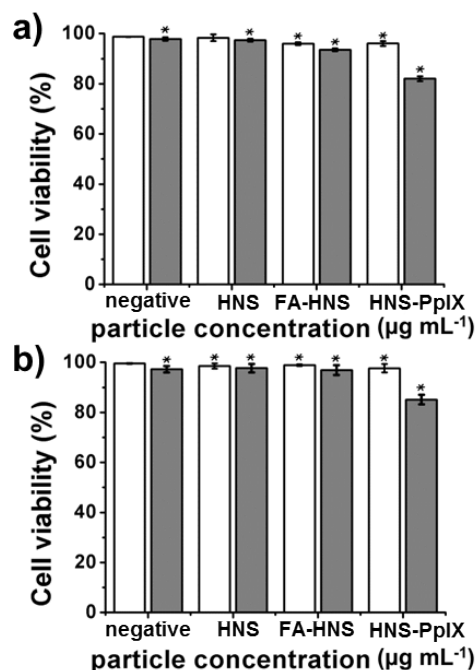


Fig. S13 Cell viability of a) MCF-7 and b) SK-BR-3 cells with different particles (HNSs, FA-HNS and HNS-PpIX were treated at the equivalent $5 \mu\text{g mL}^{-1}$ of HNS concentration). Cells were irradiated for 15 min after 24 h incubation. Values exhibit mean $\pm$ SD and each experiment was performed in triplicate. *Statistically significant difference from negative control. (white: no irradiation, gray: 15 min irradiation).

The viability of the particle treated cells was determined by trypan blue exclusion method. Viable cells with an intact plasma membrane were not stained, while dead cells became intensely blue.¹⁵ The cells were cultivated with each particle for 24 h and irradiated with visible light. The control experiment conducted for proving that HNSs, FA-HNS, and HNS-PpIX did not induce significant cell death toward MCF-7 and SK-BR-3 with 15 min irradiation (in Fig. S13). However, in this experiment, FA-HNS-incubated MCF-7 cells were slightly less viable than SK-BR-3 cells, indicating that the FA-HNS selectively acted on the FR+ MCF-7 cells. These data demonstrated that the targeting capability of the FA-HNS-PpIX to FR+ MCF-7 cells. These results indicated an effective and selective tumor targeting ability of FA and low dark toxicity of HNS, FA-HNS, and HNS-PpIX.

Intercellular H₂O₂ generation

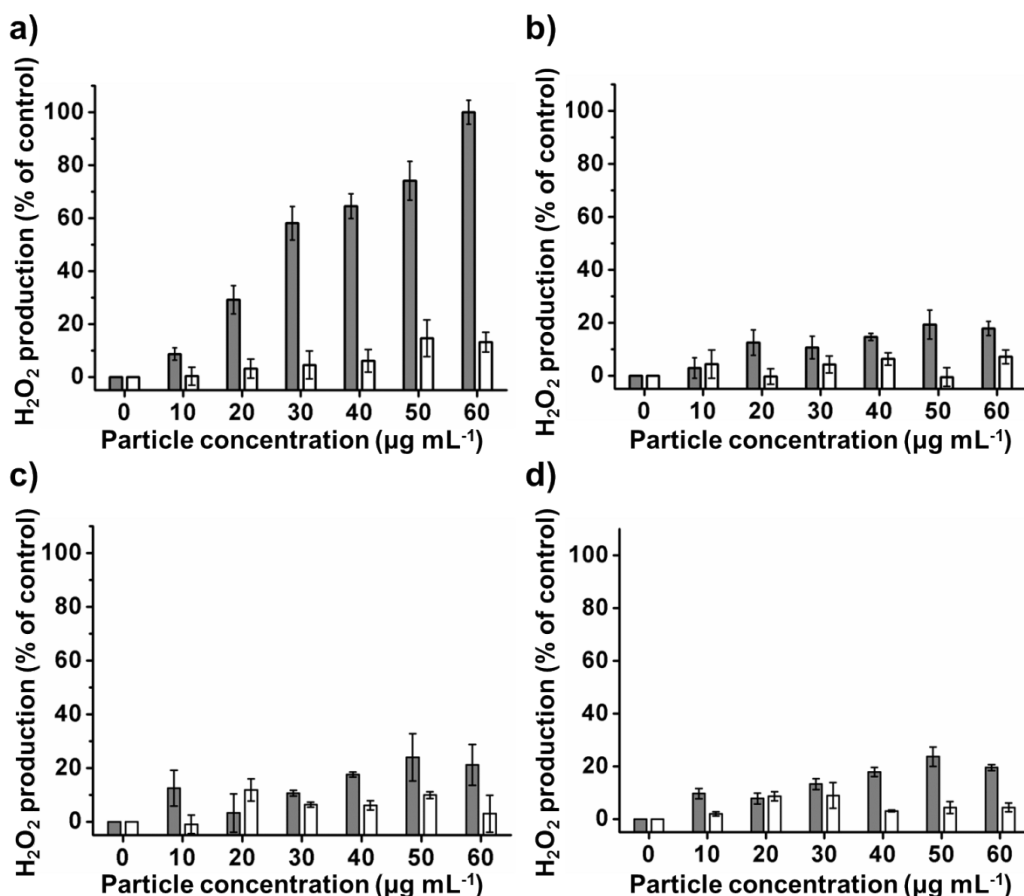


Fig. S14 H₂O₂ production by MCF-7 and SK-BR-3 cells after being incubated different concentration of the particles for 24 h. The cells were exposed to with and without irradiation condition. MCF-7 cells were incubated with a) FA-HNS-PpIX, and b) PpIX. SK-BR-3 cells were incubated with c) FA-HNS-PpIX, and d) PpIX (grey: 15 min of irradiation, white: without irradiation).

The intercellular reactive oxygen species (ROS) was detected by H₂DCFDA dye. In this manuscript, PDT induced total cell damage (including mitochondrial damage) was investigated by H₂DCFDA. The H₂DCFDA was oxidized by intercellular ROS and emitted green fluorescence.¹⁵ In general, H₂DCFDA was used for determining intercellular ROS generation. The FA-HNS-PpIX induced cell damage could be quantified by this generated H₂DCFDA. In Fig.

4a, the ROS generation was increased proportionally with FA-HNS-PpIX concentration and irradiation time. There was insignificant H₂DCFDA fluorescence change in SK-BR-3 cells. In this respect, FA-HNS-PpIX was ineffective for folate receptor negative SK-BR-3 cells. Furthermore, only PpIX treated cells (both MCF-7 and SK-BR-3) shows low cell damage levels indicating that raw PpIX is ineffective on both cell lines even with 15 min of irradiation.

Observation of mitochondrial membrane potential alteration

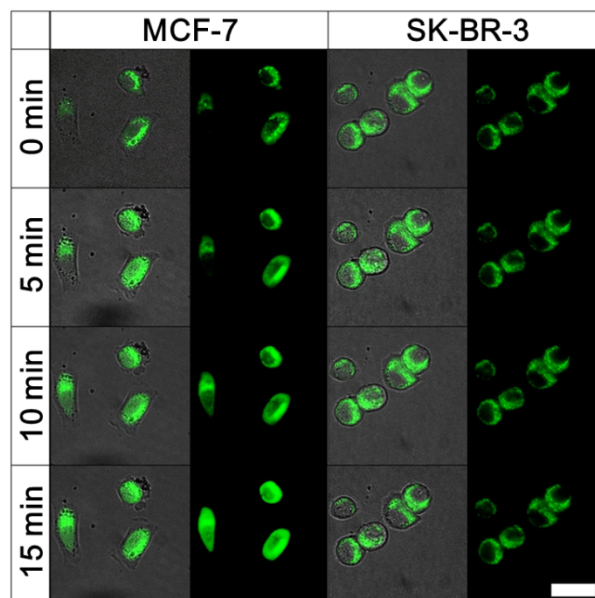


Fig. S15 Livecell fluorescent images of MCF-7 cells and SK-BR-3 cells. Cells were stained with Rh-123 for visualizing the mitochondrial membrane potential of cells. Both cells were treated with $60 \mu\text{g mL}^{-1}$ concentration of FA-HNS-PpIX irradiation time is 15 min after 24 h incubation (Scale bars: $30 \mu\text{m}$).

PDT generates $^1\text{O}_2$ and prompts a mitochondrial membrane potential integrity alteration.¹⁷ The cell damage and death can be caused by the mitochondrial dysfunction. To demonstrate the PDT effect on both cell lines, mitochondrial function was monitored by the changes of the Rhodamine-123 (Rh-123) fluorescence intensity. Rh-123 is a mitochondrial specific dye and used to investigate the effect of FA-HNS-PpIX on the mitochondrial membrane potential when light was irradiated.¹⁸ Through live cell fluorescence image in a time lapse manner, the decrease of mitochondrial membrane potential was observed with $60 \mu\text{g mL}^{-1}$ of FA-HNS-PpIX at different irradiation time scale (0, 5, 10, and 15 min). In Fig. S15, noticeable fluorescence intensity increment and uniformization of the intensity were observed in MCF-7 cells, while in SK-BR-3 cells, there was no significant fluorescence intensity change. Depolarization of the mitochondrial membrane potential induced the increase in the Rh-123 fluorescence intensity.¹⁹ This depolarization might be followed by a cell injury step: $^1\text{O}_2$ generated from PDT leads to mitochondrial membrane potential damage, resulting in mitochondrial membrane potential depolarization.²⁰ This result is strongly supported by the cell viability test (Fig. 3d, S8, and S13),

which is based on the high phototoxicity of FA-HNS-PpIX. Additionally, the heterogeneous Rh-123 fluorescence signal changed into a uniform fluorescence signal, indicating that the Rh-123 was released from the mitochondria and relocated to the cytosol because of cell damage. From these results, it can be concluded that FA-HNS-PpIX have selectively act on FR+ MCF-7 cell and effectively destruct specific type of cancer cell.

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