Electronic Supplementary Information

Enhanced cellular uptake and tumor penetration of nanoparticles by imprinting the "hidden" part of membrane receptors for targeted drug delivery

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Contents

1. Abbreviations

MIP: Molecularly imprinted polymer
NPs: Nanoparticles
MIPNPs: Molecularly imprinted polymeric nanoparticles
NIPNPs: Non-imprinted nanoparticles
FH-MIPNPs: Synthetic polymeric NPs prepared using Fn14 helical peptide as the imprinting template
FS-MIPNPs: Synthetic polymeric NPs prepared using Fn14 helical scrambled peptide as the imprinting template
CH-MIPNPs: Synthetic polymeric NPs prepared using claudin-4 helical peptide as the imprinting template
Nil: Nile red
DAPI: 4',6'-Diamidino-2-phenylindole
FOA: fluorescein o-acrylate
TB: Typan blue
TFE: 2,2,2-Trifluoroethanol
FP: Fluorescence polarization
PDT: Photodynamic therapy
ROS: Reactive oxygen species
H&E: Hematoxylin and eosin
CD: Circular dichroism
DLS: Dynamic light scattering
TEM: Transmission electron microscopy
CLSM: Confocal laser scanning microscope
AAm: Acrylamide
TBAm: N-t-butylacrylamide
BIS: N,N'-methylenebisacrylamide
APMA: N-(3-aminopropyl)methacrylamide hydrochloride
APS: Ammonium persulfate
TEMED: N,N,N',N'-tetramethylethylenediamine
HB: Hypocrellin B
IR-783:2-[2-[2-Chloro-3-[2-[1,3-dihydro-3,3-dimethyl-1-(4-sulfobutyl)-2H-indol-2-ylidene]-ethylidene]-1-cyclohexen-1-yl]-ethenyl]-3
FITC: Fluorescein isothiocyanate
DCFH-DA: 2',7'-dichlorofluorescein diacetate
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidy

2. Materials

The following materials were obtained from commercial sources:
acrylamide (AAm), N,N'-methylenebisacrylamide (BIS), N-t-butylacrylamide (TBAm), ammonium persulfate (APS), 2,2,2-trifluoroethanol (TFE), fluorescein isothiocyanate (FITC), 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI), Nile red (Nil) and Typan blue from Aladdin (Shanghai, China); N-(3-aminopropyl)methacrylamide hydrochloride (APMA) from Accela ChemBio Co. Ltd. (Shanghai, China); N,N,N',N'-tetramethylethylenediamine (TEMED) from ACROS (Beijing, China); filipin, chlorpromazine, fluorescein o-acrylate (FOA) from Sigma-Aldrich Co. LLC. (USA); Clchicines from Dingguo Changsheng Biotechnology Co. Ltd (Beijing, China); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Notlas Biology Technical Co. Ltd. (Beijing, China); 2-[2-[2-chloro-3-[2-[1,3-dihydro-3,3-dimethyl-1-(4-sulfobutyl)-2H-indol-2-ylidene]-ethylidene]-1-cyclohexen-1-yl]-ethenyl]-3,3-dimethyl-1-(4-sulfobutyl)-3H-indolium (IR-783) from Fanbo Biochemicals Co. Ltd. (Beijing, China); hypocrellin B (HB) from Yuanye Bio-Technology Co., Ltd. (Shanghai, China); and 2',7'-dichlorofluorescein diacetate (DCHF-DA) from Heowns Biochemical Technology Co. Ltd. (Tianjin, China). All other chemicals were of analytical grade.

We purchased the human prostate carcinoma cell line PC-3, human pancreatic cancer cell line BxPC-3, mouse fibroblast NIH 3T3 cell line, and human breast cancer cell BT-474 from KeyGEN Biology Co. (Nanjing, China). The cells were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin. Cultures were maintained under a humidified atmosphere of 5% CO_2 at 37℃.

BALB/c nude mice (4–6 weeks old) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). The mice were maintained under specific pathogen-free conditions. All animal experiments were performed in accordance with guidelines approved by the ethics committee of the College of Pharmaceutical Sciences, Southwest University.

3. Experimental Section

3.1 Peptide synthesis

Peptides were synthesized by solid-phase peptide synthesis performed using Fmoc-protected amino acids, and the peptides were purified using reversed-phase HPLC to homogeneity. Peptide purity (>90%) and molecular weight were ascertained by analytical HPLC and ESI mass spectrometry. The peptide sequences were as follows:
FH: ILGGALSLTFVLGLLSGFLWVRR;
FS: VTGALLWSGSGGLLIFLVFLLRR;
CH-3: MIVAGVVFLLAGLMVIVPVSWRR;

3.2 CD spectroscopy

The CD spectra of peptides were collected from 190 to 250 nm on a Bio-Logic (MOS500) spectropolarimeter with a path length of 1 mm at room temperature. The samples of FH and CH-3 were prepared at 0.3 $mg \cdot mL^{-1}$ in TFE (10%, 25%, 50%, 75%, 100%, v/v) or in EtOH (100%, v/v), respectively. The CD spectra were plotted as mean ellipticity (degree) versus wavelength λ (nm) (Figure 1A, Figure S1, Figure S10).

The template structural integrity is important to protein/peptide imprinting.[1] Trifluoroethanol (TFE) is a typical helix-stabilizing agent and can mimic hydrophobic membrane-like environment.[2] Therefore, TFE was used as a functional co-solvent in this study to maintain natural conformation of the transmembrane peptide template. However, pure TFE or too high ratio of TFE in TFE/H_2O mixtures will significantly inhibit the polymerization reaction in molecular imprinting and thus screening a proper ratio of TFE is necessary (Figure 1 and Figure S10). The 210/220 ratios or 208/222 ratios can be used as a gauge of α-helicity,[3] therefore, the ratio value may be considered as an evaluating indicator for optimizing TFE-containing reaction system. The improper 208/222 ratio may mean there are loss of structural integrity or aggregation in peptide templates, and the corresponding nanoparticles were expected to partially lose or lack target specificity.

3.3 Preparation of nanoparticles[4]

The molecularly imprinted nanoparticles (MIPNPs) were synthesized as follows: AAm (27 mg), TBAm (16 mg) and BIS (11 mg) were dissolved in ultrapure water (2.5 mL). Peptide (0.6 mg) was dissolved in 2.5 mL of TFE before addition to the monomer solution. Resulting solutions were degassed in a sonication bath under vacuum for 10 min, and then nitrogen was bubbled through the reaction mixture for

30 min. Following the addition of ammonium persulfate aqueous solution (60 mg·mL^{-1}, 100 μL) and N,N,N',N'-tetramethylethylenediamine (10 μL), the polymerization was carried out at 25°C overnight under a nitrogen atmosphere. To remove the peptide template, surfactants, and unreacted monomers, the NPs were dialyzed against 50% ethanol/water and pure water sequentially (>4 days) after the completion of polymerization. Then the purifed NPs were lyophilized. The NPs can also be purified by gel filtration chromatography using Sephadex LH-20 column.

Non-imprinted NPs (NIPNPs) were synthesized by following the same steps, except that the peptide was not added. The positively charged MIPNPs or NIPNPs were synthesized by adding APMA (1 mg),[5] and the Nil-loaded NPs (MIPNP/Nil or NIPNP/Nil) or HB-loaded NPs (MIPNP/HB or NIPNP/HB) or FOA-loaded NPs (MIPNPs/FOA or NIPNPs/FOA) were synthesized by adding Nil or HB or FOA into TFE solutions, respectively.

3.4 Characterization of NPs

The size and zeta potential of NPs in aqueous solution (1 mg·mL^{-1}) were measured using dynamic light scattering (DLS) (Zetasizer Nano ZS). The temperature of the NPs was controlled at 25°C ± 0.1°C (Figure S2A). The microstructure of NPs was examined using transmission electron microscopy (TEM) and the negative-stain method. The NPs were dissolved in ultrapure water and dropped on a carbon-coated copper grid. After air-drying, 1% phosphomolybdic acid hydrate was dropped on the carbon-coated copper grid, followed by air-drying. Then, the samples were observed in the bright-field mode at an operating voltage of 200 kV (Figure S2B).

3.5 Interaction between MIPNPs and the template

The fluorescence polarization (FP) method was used to examine the direct interaction between MIPNPs and peptides. FITC-labeled FH peptide (FITC-FH) was synthesized and purified as follows: FH (2.22 mg) and FITC (0.8 mg) was dissolved in dimethyl sulfoxide (DMSO), followed by adding N-methylmorpholine (20 μL) into the mixture. Then, the mixture was reacted at 25°C in the dark overnight. The reaction solution was purified by semi-preparative HPLC and lyophilized for later use.

FP dose-response analysis of the interaction of FITC-FH with MIPNPs were carried out at 37°C. Briefly, FITC-FH peptide dissolved in TFE was slowly added into various concentrations of MIPNPs and then incubated for 2 h at 37°C in the dark.

In the FP experiments, the fluorophore was studied using specific filters (λ_{ex} = 485 nm,

λ_{em} = 535 nm) and a microplate reader (Infinite 200Pro, Tecan). FP values (P) were defined as a function of the horizontal fluorescence intensities (I_{II}) and vertical fluorescence intensities (I_{I}): $P = (I_{II} - I_{I})/(I_{II} + I_{I})$ (Figure 1B, Figure 4A).

3.6 Uptake of MIPNPs by target cells (in vitro targeting capability)

BxPC-3 cells were incubated with positively charged FH-MIPNPs/Nil or NIPNPs/Nil for 2 h in 24-well plate. Afterward, the cells were washed with PBS (0.1 M, pH 7.4) 3 times and incubated with paraformaldehyde for 0.5 h. Next, the cells were washed with PBS (0.1 M, pH7.4) 3 times and incubated with DAPI (1 μg·mL^{-1}) for 0.5 h. Then, the cells were washed with PBS three times. The fluorescence images of the cells were obtained by fluorescence microscopy. The excitation wavelength and emission wavelength of Nil were 488 nm and 580 nm, respectively, while the excitation wavelength and emission wavelength of DAPI were 360 nm and 460 nm, respectively (Figure 1D–1E). The Nil intensity of cells treated with distinct samples was also measured by flow cytometry (ACEA Novo Cyte, 2060R). The flow velocity was 14 μL·min^{-1}, and the diameter of the sample flow was 7.7 μm. Cell-associated fluorescein was excited using an argon laser (488 nm), and fluorescence was detected at 580 nm (Figure 1C).

The fluorescence signal of positively charged CH-3-MIPNPs/Nil or NIPNPs/Nil uptake by the human prostate PC-3 cancer cells (Figure 4B), or that of FH-MIPNPs/Nil or NIPNPs/Nil by BT-474 cells were performed as mentioned above (Figure S3).

The endocytosis of NIPNPs/FOA, FS-MIPNPs/FOA or FH-MIPNs by BxPC-3 cells were performed as mentioned above, except that the extracellular FOA was quenched with trypan blue (Figure 1F–1G). [6]

3.7 Mechanism study of cellular uptake

In order to study the cellular uptake mechanism of FH-MIPNPs, BxPC-3 cells were firstly incubated with various endocytosis inhibitors including filipin (2.5 or 5 μg·mL^{-1}), chlorpromazine (6.25 or 12.5 μg·mL^{-1}), colchicine (37.5 or 70 μg·mL^{-1}) for 30 min in 24-well plate, respectively. FH-MIPNPs/Nil or NIPNPs/Nil were added and incubated for further 2 h. Next, the cells were washed with PBS (0.1 M, pH 7.4) 3 times and incubated with DAPI (1 μg·mL^{-1}) for 0.5 h. Then, the cells were washed with PBS three times. The fluorescence images of the cells were obtained by fluorescence microscopy. The excitation wavelength and emission wavelength of Nil were 488 nm and 580 nm, respectively, while the excitation wavelength and emission

wavelength of DAPI were 360 nm and 460 nm, respectively (Figure S4). Energy dependence experiments were performed by preincubating the cells at 4 ℃ for 0.5 h and FH-MIPNPs/Nil or NIPNPs/Nil were added and incubated for further 2 h at 4℃.[7]

3.8 Animals and tumor model

The subcutaneous tumor models were established by subcutaneous injection of BxPC-3 cells (4×10^6) and NIH 3T3 cells (2×10^6)[8] into the right striatum (1.8 mm lateral and 0.6 mm anterior to bregma at 3 mm depth) of BALB/c nude mice. The treatment of model nude mice was initiated after the tumor had been grown to 50–100 mm^3 in size. Tumor volume was calculated using the following formula: tumor volume = [length × $(width)^2$]/2. The length and width of each tumor were determined using vernier calipers.

The BT-474 tumor models were established as mentioned above.

3.9 In vivo targeting efficacy test

Once tumor volume reached 50–100 mm^3, the BxPC-3/NIH 3T3 tumor-bearing nude mice were randomly divided into 2 groups. The model nude mice were injected with 100 μL of the positive charged FH-MIPNPs/IR-783 or NIPNPs/IR-783 via the tail vein. The total dose of NPs in each mouse was 50 $mg \cdot kg^{-1}$ with the same concentration of IR-783 of 20 $\mu g \cdot mL^{-1}$ (Figure 2). The model nude mice were anesthetized by intraperitoneal injection of 10% chloral hydrate and assessed by an in vivo imaging system (FX Pro, FX-Pro, BRUKER, Germany) with an excitation bandpass filter at 730 nm and an emission wavelength of 790 nm at preset time points (BxPC-3/NIH 3T3 group: 1 h, 2 h, 4h, 8 h, and 24 h).

The targeting ability of MIPNPs and NIPNPs in BT-474 tumor models performed as mentioned above (Figure S5).

3.10 In vivo tumor penetration study of nanoparticles

BALB/c nude mice bearing the Bxpc-3/NIH 3T3 tumors were injected (iv) free Nil, FH-MINPs/Nil, and NIPNPs/Nil. After 4 h, mice were sacrificed and the excised tumors were snap-frozen immediately in optimum cutting temperature (OCT) medium at -80 ℃. The tumor sections (5 um) were prepared, air-dried for 10 min, and fixed with 4% paraformaldehyde for 10 min. The slices were observed using confocal microscopy, and five random fields were selected to measure the penetration depth of Nil (Figure 3A).

3.11 3D tumor spheroids-based study

To evaluate the tumor penetration ability of MIPNPs in vitro, core-shell 3D tumor spheroids was generated, in which tumor cells (BxPC-3) were enclosed by fibroblasts (NIH 3T3) to simulate the in vivo condition.[9] BxPC-3 cells (1×10^4) were suspended in the ultra-low attachment round bottom 96 well plates(Costar, Corning, NY) to form BxPC-3 multicellular spheroids, The handing drops were centrifugated at a speed of 900 rpm for 2 min to cluster the cells to the bottom of the wells, and then the resulting cellular aggregates were incubated at 37℃ for 3 days. On the fourth day, NIH 3T3 cells (1×10^4) were seeded into each well, and then the core-shell tumor spheroid was incubated at 37℃ for another 2 days. When the diameter reached about 400 μm, core-shell 3D tumor spheroids were used to evaluate drug penetration. For the time-lapse assay, MINPs/FOA and NIPNPs/FOA were added into different wells, and incubated with 3D spheroids in 37℃ for 1, 2, 4, 8, 12, 24 h. CLSM was used to image the spheroid at determined time points approximately 150 μm from the bottom (Figure 3B).[10]

3.12 Cellular reactive oxygen species detection during irradiation

After the BxPC-3 cells were incubated with the positively charged FH-MIPNPs/HB or NIPNPs/HB, they were further incubated with 10 μM DCFH-DA for 1 h and irradiated with a 450-nm laser at a power of 100 $mW \cdot cm^{-2}$ for 10 s for fluorescence detection of DCF with by flow cytometry,[11] which could give the level of intracellular reactive oxygen species (ROS) (Figure S6).[12]

3.13 In vivo anti-tumor study

BALB/c nude mice bearing the Bxpc-3/NIH 3T3 tumors were divided into 3 groups (six mice per group). Mice of each group were treated intravenously with positively charged FH-MIPNPs/HB (50 $mg \cdot mL^{-1}$, 100 μL), positively charged NIPNPs/HB (50 $mg \cdot mL^{-1}$, 100 μL), or PBS (0.1 M, pH 7.4) on Day 0 and Day 5, respectively. Subsequently, the tumor of mice were illuminated locally with 650-nm laser light for 10 min (800 $mW \cdot cm^{-2}$), considering that 650 nm penetrate more deeply into tissue than 450 nm.[13,14] Tumor growth in all animals were monitored every 2 days during the complete survey (Figure S7).[15]

3.14 Cytotoxicity of nanoparticles

The in vitro cytotoxicity of BxPC-3 cells was determined by measuring cell viability using the iCelligence system (Acea Biosciences). The instrument of iCelligence system measures cell status (given as cell index) in the form of electrical impedance, which is determined by cell morphology, cell adhesion, and cell viability. As cells

attach to the bottom of the plate coated with electrodes, a change in local ionic environment occurs, resulting in increased impedance. Measurements were carried out according to the instructions of the supplier. After seeding 300 μL of the suspension of 15,000 BxPC-3 cells into 8-well E-plates, the cell index was recorded every 1 min for about 12 h for the cells to reach the log phase. The cells were then exposed to 2, 4, or 8 mg·mL-1 of the positively charged NPs, and the cell index was read again for additional 100 h (Figure S8A).[16-17]

Effects of FH-MIPNPs on BxPC-3 viability measured by the MTT assay were performed as follows. Bxpc-3 cells (5×10^3) were seeded in 96-well plates and incubated for 12 h before being treated with various concentrations FH-MIPNPs (1, 2 4, 8 $mg \cdot mL^{-1}$) for 48 h. After removing the medium, cells were washed with PBS for 3 times, and then 20 μL of MTT (5 $mg \cdot mL^{-1}$ in PBS) was added into each well and incubated at 37°C for 4 h. After the culture medium was removed, 150 μL of DMSO was added into each well and mixed for 15 min in the dark at room temperature, and then measured for optical density (OD) values at 490 nm. Cell viability was expressed as the percentage of the OD value of control cells from treated cells (Figure S8B).

3.15 In vivo toxicity test

To detect the in vivo toxicity of NPS, positively charged FH-MIPNPs (300 $mg \cdot kg^{-1}$) were injected into the nude mice. The control group mice were injected with an equal volume of physiological saline. The body mass of the mice was recorded daily, and the mice were sacrificed in the first 2 weeks after surgery. The primary internal organs (heart, liver, spleen, lung, kidney) were removed and stored in paraformaldehyde (4%) for histological analysis by hematoxylin and eosin (H&E) staining (Figure S9).

4. References

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5. Supporting figures

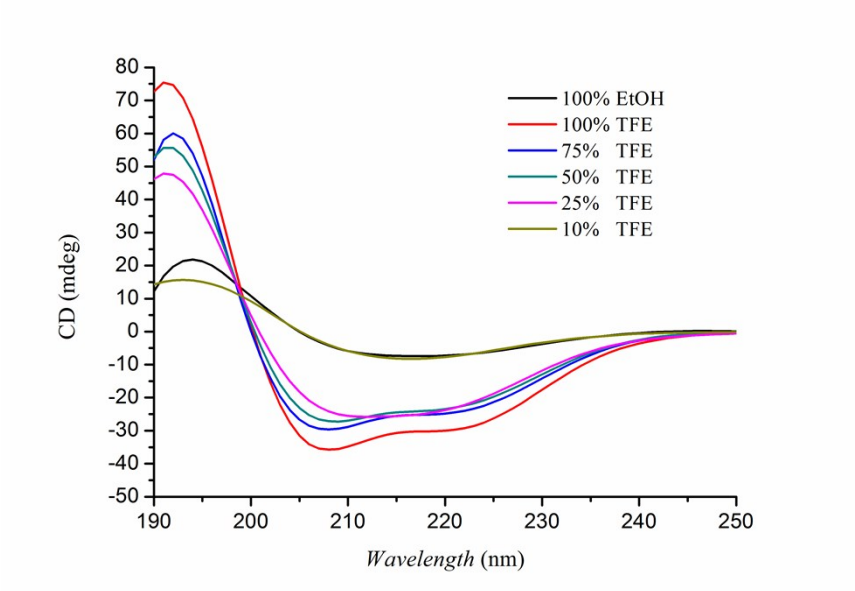

Figure S1. The CD spectra of the FH peptide (0.3 mg·mL^{-1}) in TFE (10%, 25%, 50%, 75%, 100%) and ethanol at 25°C.

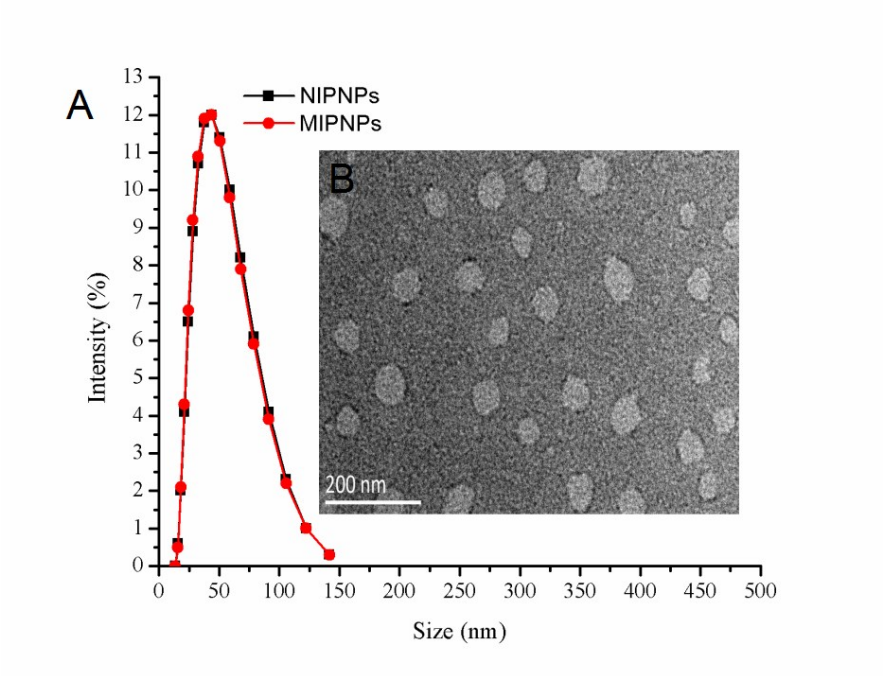

Figure S2. (A) The particle size and distribution of positively charged FH-MIPNPs determined using DLS. (B) A TEM image of positively charged FH-MIPNPs.

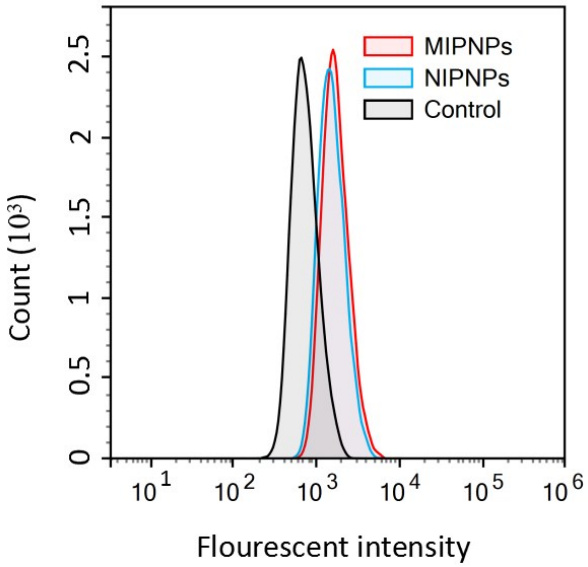

Figure S3. Flow cytometric detection of the cellular uptake of Nil-loaded NPs by BT-474 cells. FH-MIPNPs/Nil (red), NIPNPs/Nil (blue), and control solution (black).

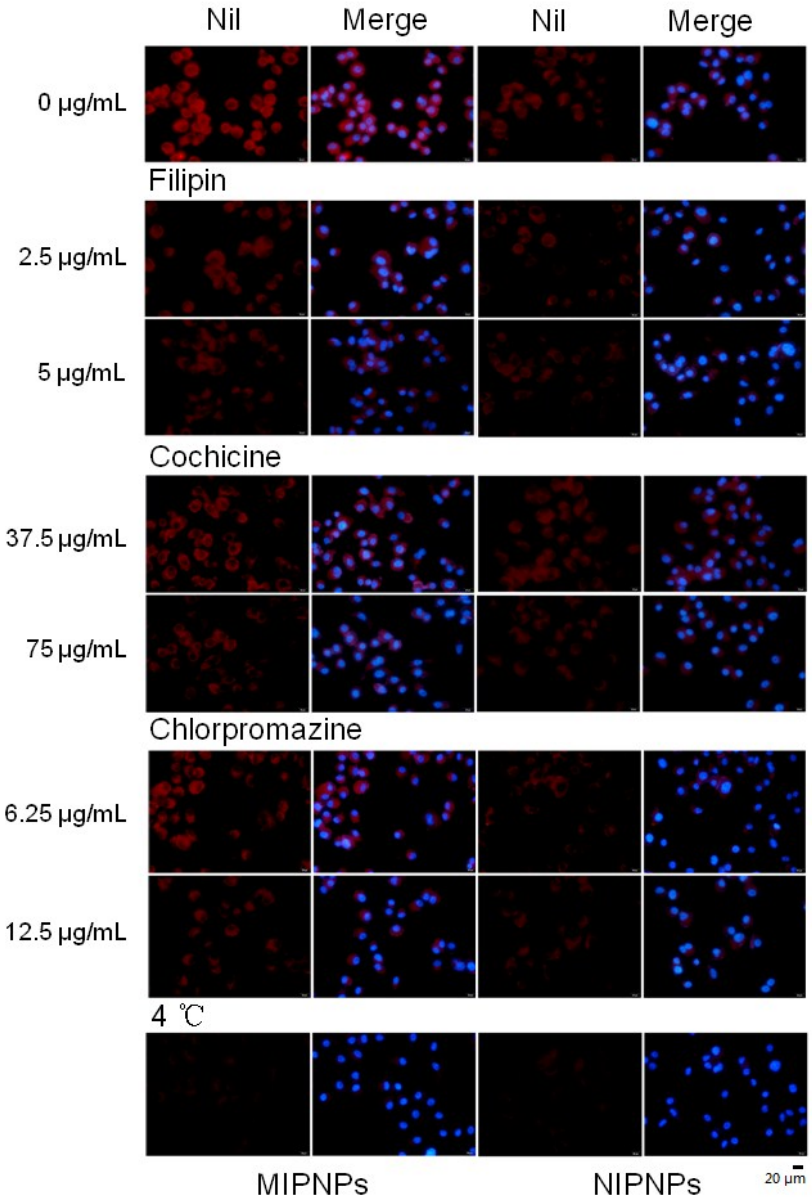

Figure S4. Fluorescence images of uptake mechanism study of positively charged MIPNPs or NIPNPs by BxPC-3 cells. MIPNPs: FH-MIPNPs/Nil; NIPNPs: NIPNPs/Nil. Nil uptake is represented by red fluorescence.

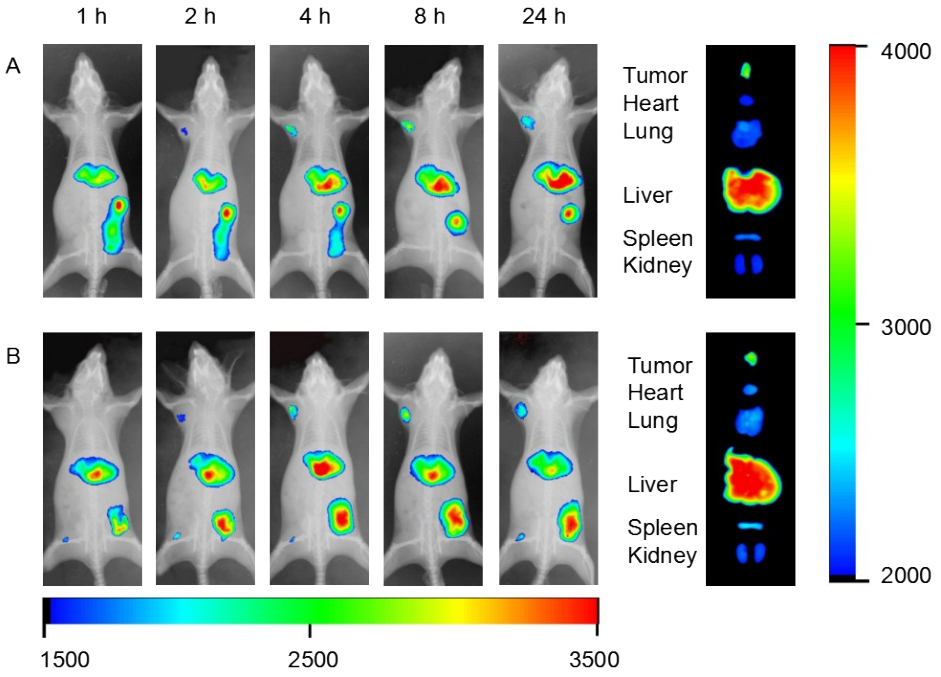

Figure S5. In vivo distribution of nanoparticles in BT-474-tumor-bearing nude mice. Model mice were injected with MIPNPs (A) and NIPNPs (B, 100 uL, 50 mg·kg^{-1}) through the tail vein. The mice were examined using an in vivo imaging system at indicated time points, and ex vivo organs were examined after 24 h.

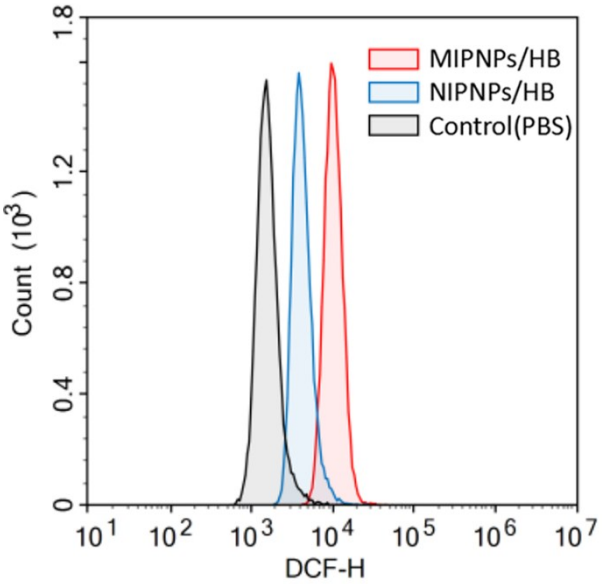

Figure S6. Flow cytometric detection of ROS generated during PDT performed after adding positively charged FH-MIPNPs/HB (red), positively charged NIPNPs/HB (blue), or control solution (black); the ROS probe DCFH-DA was used for ROS detection.

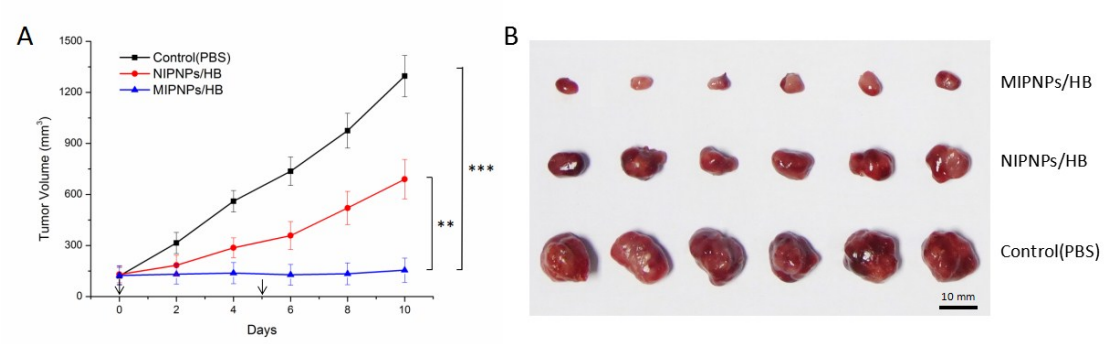

Figure S7. In vivo anti-tumor study. (A) Tumor volume growth curves of BxPC-3-tumor-bearing nude mice treated with positively charged FH-MIPNPs/HB, positively charged NIPNPs/HB or control (PBS); $^{**}p < 0.01$ and $^{***}p < 0.005$. (B) Digital photos showing the morphology of the tumor in three groups.

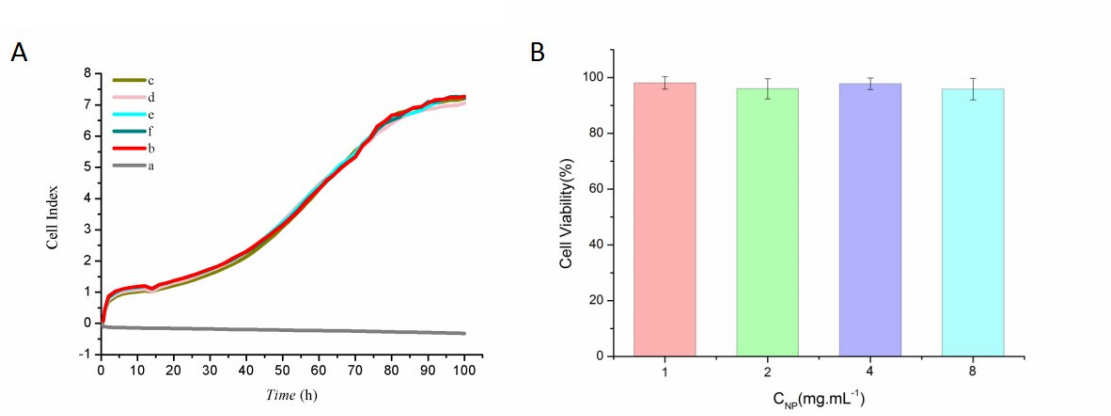

Figure S8. In vitro cytotoxicity of MIPNP-F in BxPC-3 cells. (A) Real-time measurement of BxPC-3 cell growth (cell index) following treatment with various concentrations of positively charged FH-MIPNPs (2, 4, 8 mg·mL^{-1}) by a real-time viability assay (xCelligence). Lines are the means of 3 replicates. (a): blank (no cell); (b): BxPC-3; (c): cell medium; (d): 2 mg·mL^{-1} of NPs; (e): 4 mg·mL^{-1} of NPs; (f): 8 mg·mL^{-1} of NPs. (B) Effect of FH-MIPNPs on viability of BxPC-3 measured by the MTT assay.

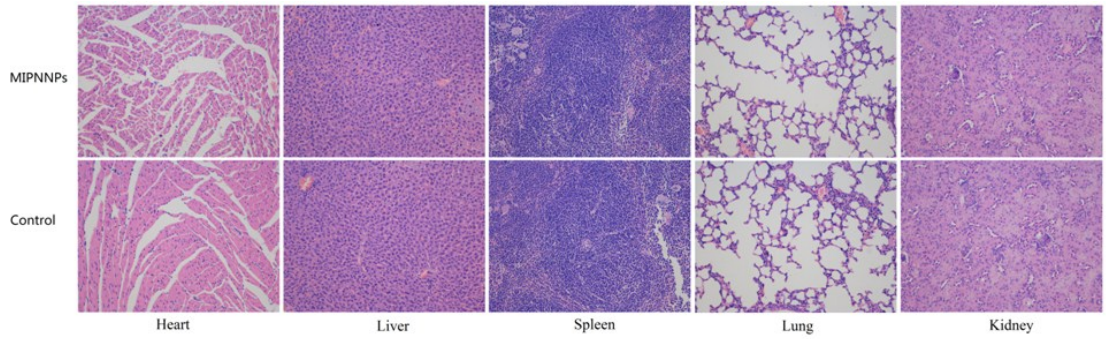

Figure S9. Tissue sections examined for in vivo toxicity of positively charged NPs. Hearts, livers, spleens, lungs, and kidneys were obtained 2 weeks after intravenous injection of MIPNPs or physiological saline (dose = 300 $mg \cdot kg^{-1}$). Organ sections were stained with H&E and examined at 200× magnification.

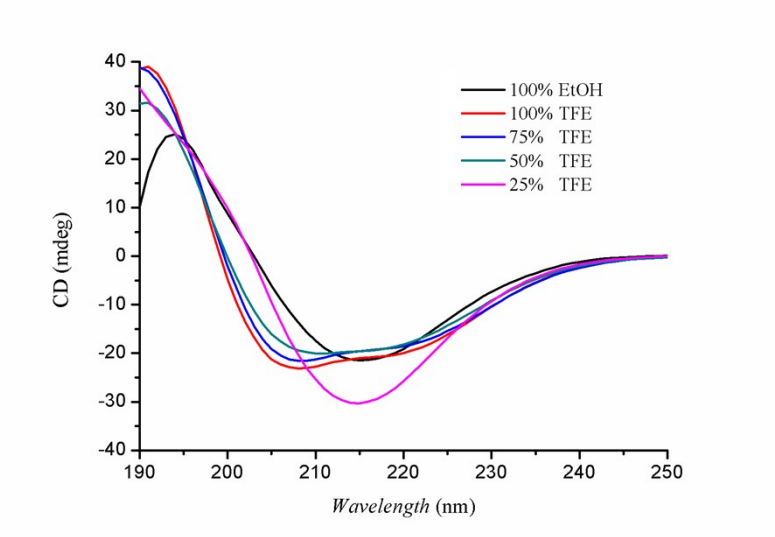

Figure S10. The CD spectra of the CH-3 peptide (0.3 mg·mL^{-1}) in TFE (25%, 50%, 75%, 100%) and ethanol at 25°C.