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Supporting Information

Responsive Peptide Nanofibers with Theranostic and Prognostic Capacity

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Experimental Procedures

1. Materials

5,15-(di-4-carboxyphenyl)-10,20-(diphenyl)porphyrin (98%) was purchased from PorphyChem, France. Fmoc-NH-PEG(6)-COOH were purchased from Iris Biotech. 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA), 2',7'-Dichlorofluorescin diacetate (DCFH-DA), N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), N,N-Diisopropylethylamine (DIPEA), N,N′-Diisopropylcarbodiimide (DIC), Fmoc-His(Trt)-OH, Oxyma Pure (ethyl 2-cyano-2-(hydroxyimino)acetate), triisopropylsilane (TIS), trifluoroacetic acid (TFA), dimethyformamide (DMF), dimethylsulfoxide (DMSO), diethylether and all other chemicals were supplied by Sigma-Aldrich. Rink Amide resin was purchased from the Rapp Polymere, Germany. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from ThermoFisher Scientific. Ultra-pure MilliQ water was obtained from a Labconco Water Pro PS purification system (18.2 ME).

2. Synthesis and Methods

2.1 Synthesis of porphyrin-peptides

Peptides were synthesized *via* Fmoc solid-phase peptide synthesis using an automated Intavis MultiPep RSi peptide synthesizer. Rink Amide resin (0.59 mmol/g loading) was used for the synthesis of peptide. Fmoc-deprotection was achieved using 20% piperidine in DMF. For $PHHPEG_6$ synthesis, Fmoc-NH-PEG(6)-COOH was dissolved in DMF and coupled to the resin using DIPEA/HBTU (2:1) as activators. Then Fmoc-His(Trt)-OH was dissolved in DMF and coupled to the resin using DIPEA/HBTU (2:1) as activators. The other Fmoc-His(Trt)-OH was coupled to the resin using the same method. After that 5,15-(di-4-carboxyphenyl)-10,20-(diphenyl)porphyrin was dissolved in DMF and coupled to the resin using DIC/OxymaPure (1:1.5) as activators. For PHH synthesis, similar methods with $PHHPEG_6$ synthesis were applied except not coupling Fmoc-NH-PEG(6)-COOH. After synthesis, resin cleavage of the protected peptides were performed using 2.5%/2.5%/95% H2O/TIS/TFA and the peptides were subsequently precipitated in ice-cold diethylether. The resultant crude solids were washed with diethylether for five times and lyophilized. The peptides were further purified using a preparative reversed-phase high performance liquid chromatography (HPLC) system which comprised of a LCQ Deca XP Max (Thermo Finnigan) ion-trap mass spectrometer equipped with a Surveyor autosampler and Surveyor photodiode detector array (PDA) detector (Thermo Finnigan). Solvents were pumped using a high-pressure gradient system using two LC-8A pumps (Shimadzu) for the preparative system and a two LC-20AD pumps (Shimadzu) for the analytical system. The crude mixture was purified on a reverse phase C18 column (Atlantis T3 prep OBD, 5 µm, 150 x 19 mm, Waters) using a flow of 20 mL/min and linear acetonitrile gradient in water with 0.1% v/v TFA. Fractions with the correct mass were collected using a PrepFC fraction collector (Gilson Inc). The peptide solutions were collected and lyophilized. The lyophilized peptides were washed with 0.1 M Na_2CO_3 and centrifuged to remove residual TFA. Then, the peptides were washed with MilliQ water for three times to remove Na_2CO_3.

2.2 Self-assembly of PHH and $PHHPEG_6$

The PHH and $PHHPEG_6$ powder were dissolved in DMSO at the concentration of 100 mg/mL, respectively. Then, 1 mL MilliQ water was added into 10 uL of PHH or $PHHPEG_6$ solutions under ultrasonication for 30 s to form PHH NPs or $PHHPEG_6$ NFs. These PHH NPs or $PHHPEG_6$ NFs were aged one day before use.

2.3 Characterization of PHH and $PHHPEG_6$ molecules

The 1H NMR spectra were recorded on a Bruker (400 MHz) spectrometer with a Bruker Sample Case auto-sampler, with DMSO-d6 as the solvent and TMS as an internal standard. Analytical LC-MS was performed on a C4, Jupiter SuC4300A, 150 × 2.00 mm column with a gradient 5%−100% acetonitrile in H_2O supplemented with 0.1% v/v formic acid (FA) in 15 min. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) measurements were performed with an Autoflex Speed (Bruker, Bremen, Germany) instrument equipped with a 355 nm Nd:YAG smartbeam laser with maximum repetition rate of 1000 Hz, capable of executing both linear and reflector modes. The accelerating voltage was held at 19 kV and the delay time at 130 nanoseconds for all experiments. Mass spectra were acquired in the reflector positive ion mode by summing spectra from 500 random laser shots at an acquisition rate of 100 Hz. The MS spectra were calibrated with CsI clusters of known masses.

2.4 Characterization of nanostructures

The croy-TEM experiments were performed using the TU/e CryoTitan (Thermo Fisher Scientific) equipped with a field emission gun and autoloader and operated at 300 kV acceleration voltage in low-dose bright-field TEM mode. Samples for cryo-TEM were prepared by glow-discharging the grids (Lacey carbon coated, R2/2, Cu, 200 mesh, EM sciences) in a Cressington 208 carbon coater for 40 seconds. Then, 4 µl of samples were pipetted on the grid and blotted in a Vitrobot MARK III at room temperature and 100% humidity. The grid was blotted for 3 seconds

(offset -3) and directly plunged and frozen in liquid ethane. Cryo-TEM images were acquired with zero loss energy filtering mode (Gatan GIF 2002, 20eV energy slit) on a CCD camera (Gatan model 794). Scanning Electron Microscopy (SEM): FEI Quanta 200 3D FEG was used to characterize the morphology of these nanostructures. Dynamic Light Scattering (DLS) experiments were performed at a Malvern Z90 Zetasizer equipped with a 633 nm He−Ne laser and an avalanche photodiode detector was used to characterize the hydrodynamic size of the particles. The scattering light at 173° angle was detected and used to analyze the size and distribution. The Zeta-potential of NFs at different pH was measured with the Malvern Z90 Zetasizer by diluting 0.2 mg/mL PHHPEG$_6$ NFs (1:10 dilution) in 0.1 mM PBS at pH 7.4, 6.3 and 5.0. The pH change of the solution was recorded using a Mettler Toledo™ FiveEasy Plus™ FEP20 pH Meter. Confocal laser scanning microscopy (CLSM) imaging of cell samples was performed using the Leica TCS 264 SP5X.

2.5 Stability of PHH NPs and PHHPEG$_6$ NFs

1 mg/mL PHH NPs and PHHPEG$_6$ NFs stock solution was diluted to 0.2 mg/mL with phosphate buffer solution at pH 7.4. 1 mg/mL PHHPEG$_6$ NFs stock solution was also diluted to 0.2 mg/mL with H_2O, DMEM, 10% FBS and DMEM+10% FBS at pH 7.4. The size of the nanostructures measured by DLS.

2.6 pH-responsiveness of PHH NPs and PHHPEG$_6$ NFs

To investigate pH-responsiveness of the PHH NPs and PHHPEG$_6$ NFs, the 1 mg/mL PHH NPs and PHHPEG$_6$ NFs stock solution was diluted to 0.2 mg/mL with phosphate buffer solution at pH 7.4, 6.3 and 5.0. The size of the nanostructures measured by DLS. The fluorescence spectra were recorded on a fluorescence spectrophotometer (*Ex:* 518 nm, *Em:* 600−800 nm, Hitachi F-4600, Japan).

2.7 Photostability and singlet oxygen generation of PHHPEG$_6$ nanostructures in solutions

The absorption spectrum of PHHPEG$_6$ NFs was measured before and after light irradiation for specific time intervals with light intensity of 100 mW/cm^2. To examine the influence of buffer pH on the photoactivity of the PHHPEG$_6$ nanostructures, the solution was diluted to 0.2 mg/mL using 0.1 M phosphate buffer solution at pH 7.4, 6.3 and 5.0 and DMSO, respectively. The singlet oxygen probe ABDA was added at a final concentration of 100 μM. The PHHPEG$_6$ nanostructures solution was then illuminated with a 660 nm laser (BeamQ Lasers) at photodensity of 0.12 W/cm^2 for 10 min. The ABDA consumption was determined by measuring the absorbance intensity of ABDA at 378 nm as a function of time by a Spark 10 M microplate reader (TECAN, Switzerland). ABDA (100 μM) in 0.1 M phosphate buffer solution at pH 7.4, 6.3 and 5.0 and DMSO under light illumination were set as the control.

2.8 Intracellular trafficking of PHHPEG$_6$ nanostructures

4T1 cells and MCF-7 cells were seeded in μ-Slide 8 Well (5 × 10^4 cells/well). After culture for 24 h, the cells were incubated with PHHPEG$_6$ NFs (200 μg/mL) at pH 7.4 and 6.3 for 24 h, and then washed three times with using PBS. Subsequently, the cells were stained with of LyosTracker Green (100 nM) (Life Technologies), endoplasmic reticulum (ER)-Tracker green (Abcam), MitoTracker green (Life Technologies) and GolgiTracker green (Abcam) for 30 min and Hoechst 33342 (10 μg/mL) (Life Technologies) for 10 min at 37 °C. Then, the cells were washed three times with PBS, resuspended in 200 μL of PBS, and immediately observed using CLSM. Flow cytometry was performed on a FACS Aria III (BD Biosciences) equipped with a 70 μm nozzle. Events representing single cells were gated based on the forward height scatter vs the forward area scatter. For each measurement, fluorescence intensities of 10000 individual cells were recorded and analyzed using custom-written MATLAB scripts. The proteolysis of PHHPEG$_6$ NFs inside 4T1 cells and MCF-7 cells was investigated by CLSM. The NFs were washed from the 4T1 and MCF-7 cells after 24 h co-culturing and the cells were cultured in medium for another 24 h. The fluorescence of PHHPEG$_6$ NFs in 4T1 cells and MCF-7 cells at 24 h and 48 h was measured by CLSM.

2.9 Singlet oxygen generation *in vitro*

4T1 cells and MCF-7 cells were seeded in μ-Slide 8 Well (5 × 10^4 cells/well). After culture for 24 h, the cells were incubated with PHHPEG$_6$ NFs (200 μg/mL) at pH 7.4 and 6.3. Cell culture medium were removed after 24 h incubation and washed three times cells with PBS. The cells were continually incubated fluorescent probe DCFH-DA (20 μM) for 30 min and Hoechst 33342 (10 μg/mL) for 10 min at 37 °C in the dark, washed three times with PBS, and resuspended in 200 μL of PBS. The cells were then treated with a 660 nm laser for 10 min at a photodensity of 0.12 W/cm^2 and examined using CLSM.

2.10 Cytotoxicity Assay *in vitro*

4T1 cells and MCF-7 cells were seeded in 96-well tissue culture plates (5 × 10^3 cells/well). After culture for 24 h and incubated with PHHPEG$_6$ NFs at different concentrations (0, 50, 100, 150, 200 μg/mL) at pH 7.4 and 6.3. After 24 h incubation, removed the cell culture medium, washed cells three times with PBS and resuspended cells in 100 μL of DMEM. The cells were then treated with a 660 nm laser for 10 min and continually cultured for an

additional 24 h. The cell viability was then analyzed by the MTT assay. The 4T1 cells and MCF-7 cells treated with $PHHPEG_6$ NFs without light illumination were set as the control.

2.11 Animal models

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care & Welfare Committee of Guangxi Medical University in compliance with Chinese law for experimental animals with an approval number of 202111007. Female BALB/c-nu mice were purchased from Laboratory Animal Center, Guangxi Medical University and were housed in an environmentally controlled animal facility with a regular 12/12 light/dark cycle. 4T1 cells were collected and suspended in PBS at a concentration of 6×10^7 cells mL^{-1}. Each mouse was injected with a 100 μL suspension solution of 4T1 cells on the hind hip. The *in vivo* experiments were carried out when the tumors reached a size of approximately 100 mm^3.

2.12 *In vivo* biodistribution analysis

The 200 μL of 2 mg/mL $PHHPEG_6$ NFs in an aqueous 5% glucose solution was injected into five 4T1 tumor bearing nude mice via the tail vein. The mice were anesthetized and scanned by an *in vivo* imaging system (CRi Maestro 2) at different treated time points as 4 h, 12 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h, 192 h, 216 h and 264 h to observe the florescence of $PHHPEG_6$. At 48 and 264 h post injection, the mice were sacrificed, and the harvested organs (liver, lung, spleen, kidneys and heart) and tumors were imaged by this imaging system. The in vivo imaging assay was performed in BLT AniView600 Multi-mode In Vivo Animai Imaging System. The fluorescence intensity was obtained using the built-in software for the imaging system.

2.13 *In vivo* antitumor prognostic monitoring

When the tumor volume reached to 100 mm^3, the mouse in each group were injected with 200 μL of 2 mg/mL $PHHPEG_6$ NFs *via* the tail vein. At 48 h post injection, the mice tumor sites were irradiated by a 660 nm laser (0.3 W/cm^2) for 10 min. In the following days, the fluorescence was measured every day.

2.14 *In vivo* antitumor efficacy

Twenty nude mice implanted with 4T1 tumor were randomly divided into four groups (n = 5). When the tumor volume reached to 100 mm^3, the mice in each group were injected with 5% glucose solution (group 1, Control), 5% glucose solution (group 2, Laser), 200 μL of 2 mg/mL $PHHPEG_6$ NFs (group 3, $PHHPEG_6$ NFs), 2 mg/mL $PHHPEG_6$ NFs (group 4, $PHHPEG_6$ NFs + laser), respectively *via* the tail vein. At 48 h post injection, the groups 2 and 4 mice tumor sites were irradiated by a 660 nm laser (0.3 W/cm^2) for 10 min. In the following 16 days, the body weight and tumor volume of all mice were measured every day. The tumor volumes were calculated by the following equation: tumor volume = (diameter × $width^2$/2). All mice were sacrificed at the 16th day post treatment and the major organs (heart, liver, spleen, lung, and kidneys) were harvested and fixed with a 4 wt% paraformaldehyde solution for the histological analysis to evaluate the biosafety of the $PHHPEG_6$ nanostructures. After embedded in paraffin and stained by hematoxylin and eosin (H&E), the organs were examined by hematoxylin-eosin (H&E) staining.

2.15 Statistical analysis

Data are expressed as mean ± standard deviation.

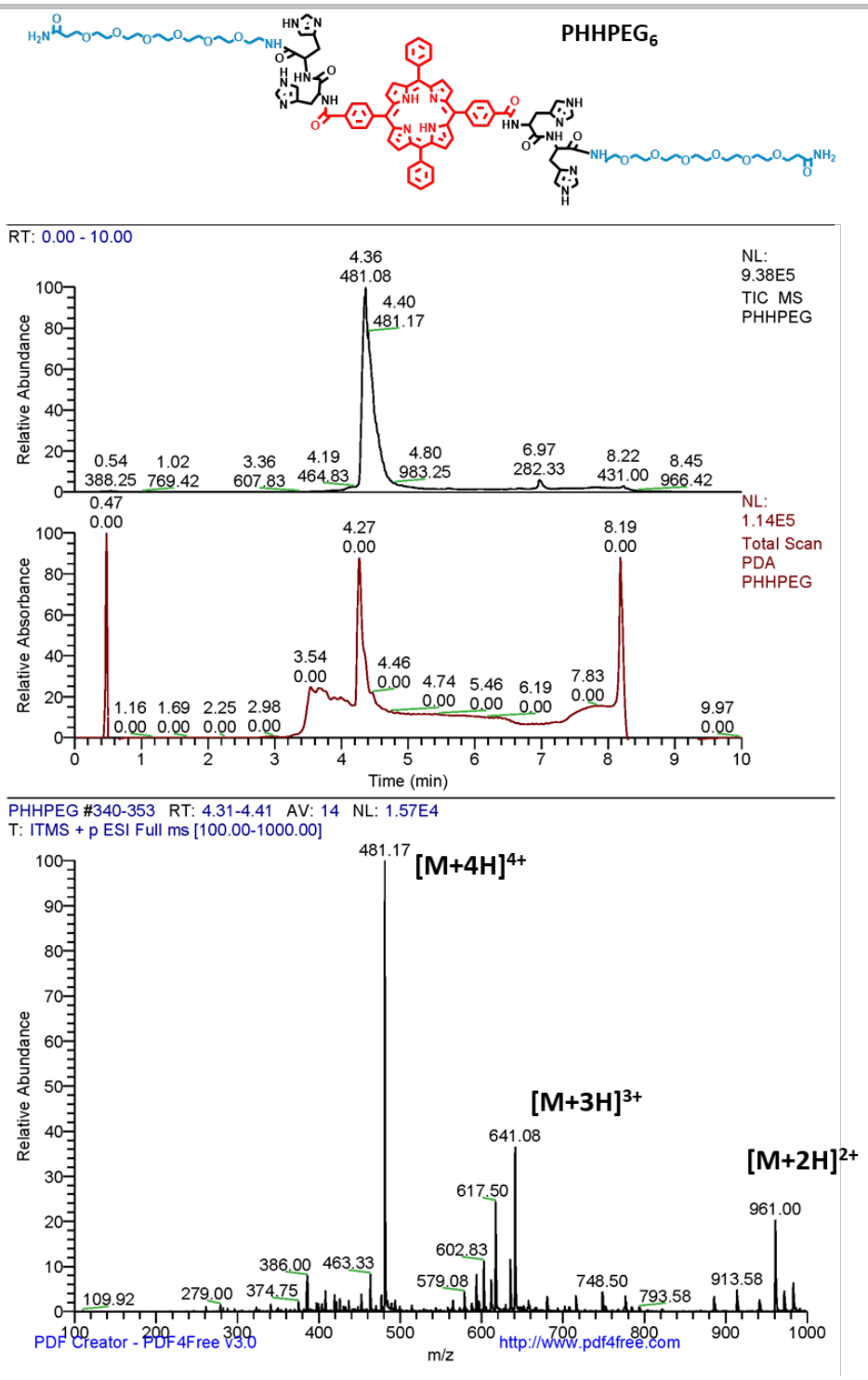

Figure S1. Chemical structure of PHHPEG$_6$ and LC-Mass analysis of PHHPEG$_6$ after purification, RT=4.31-4.41; ESI-MS: $[M+2H]^{2+}$, $[M+3H]^{3+}$, $[M+4H]^{4+}$.

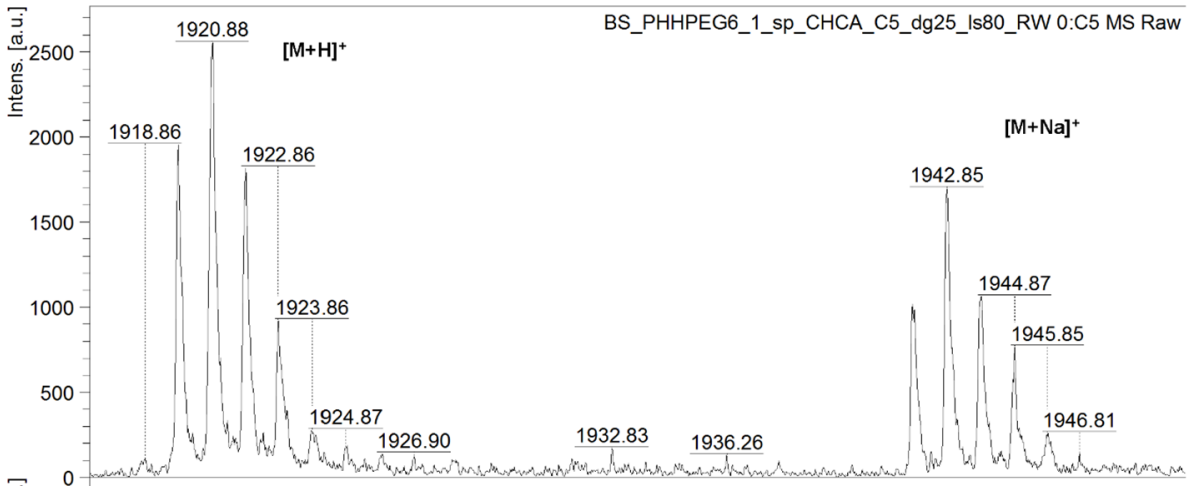

Figure S2. MALDI-TOF Mass analysis of PHHPEG$_6$. m/z, [M+H]+, 1920.88, [M+Na]+, 1942.85.

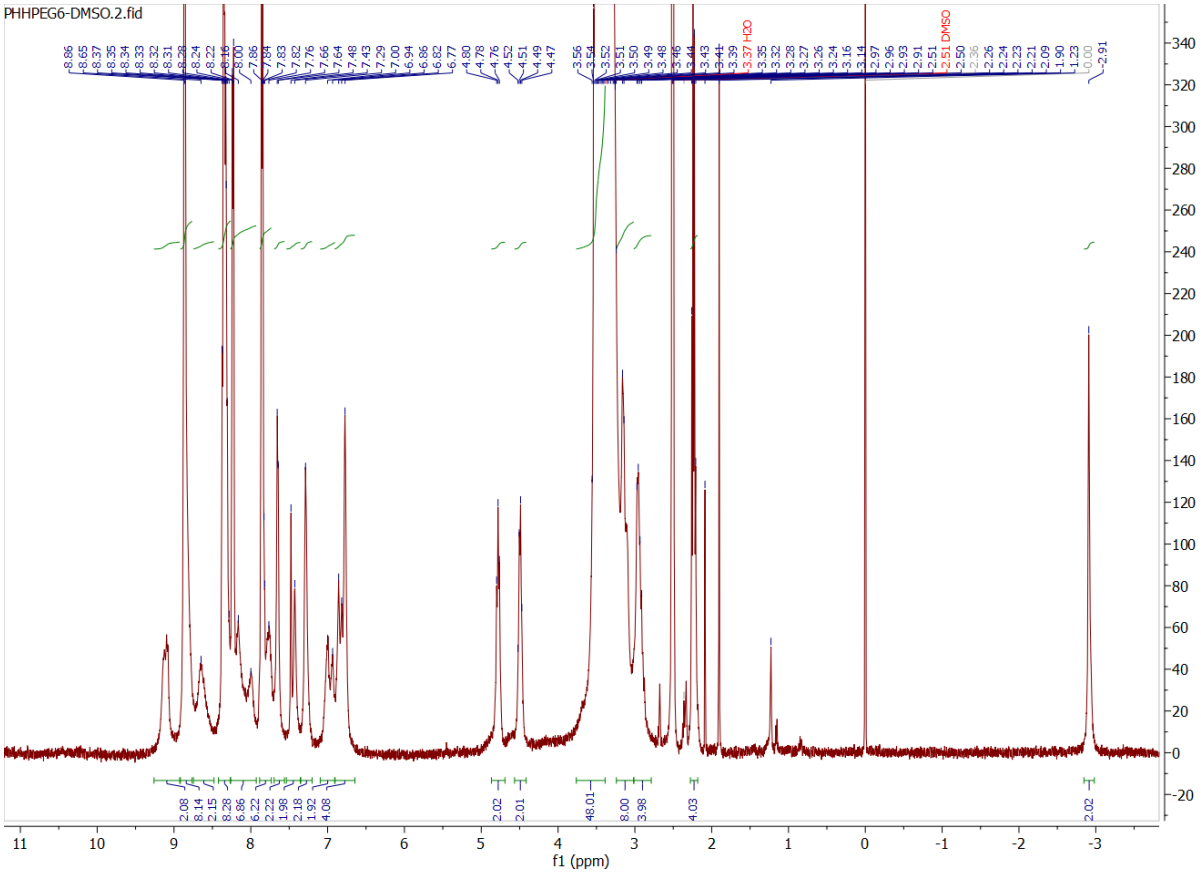

Figure S3. H-NMR of PHHPEG$_6$ molecule in DMSO-d6.

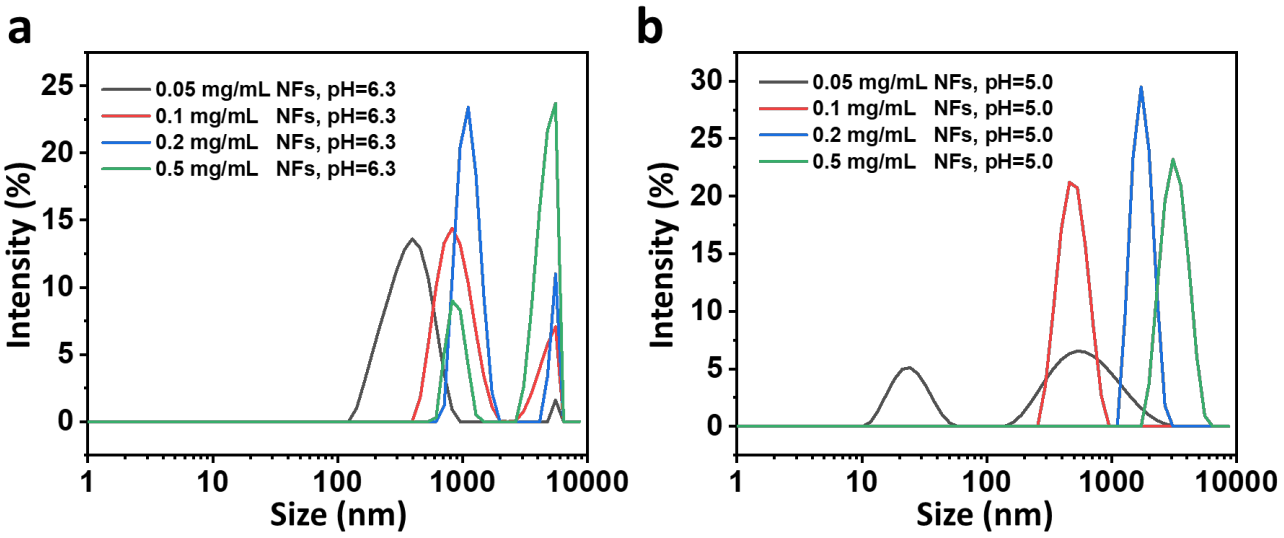

Figure S4. Size distribution of PHHPEG$_6$ NFs at different concentrations of 0.05 to 0.5 mg/mL in phosphate buffer solution at a) pH 6.3 and b) 5.0 after 1 h, measured by DLS.

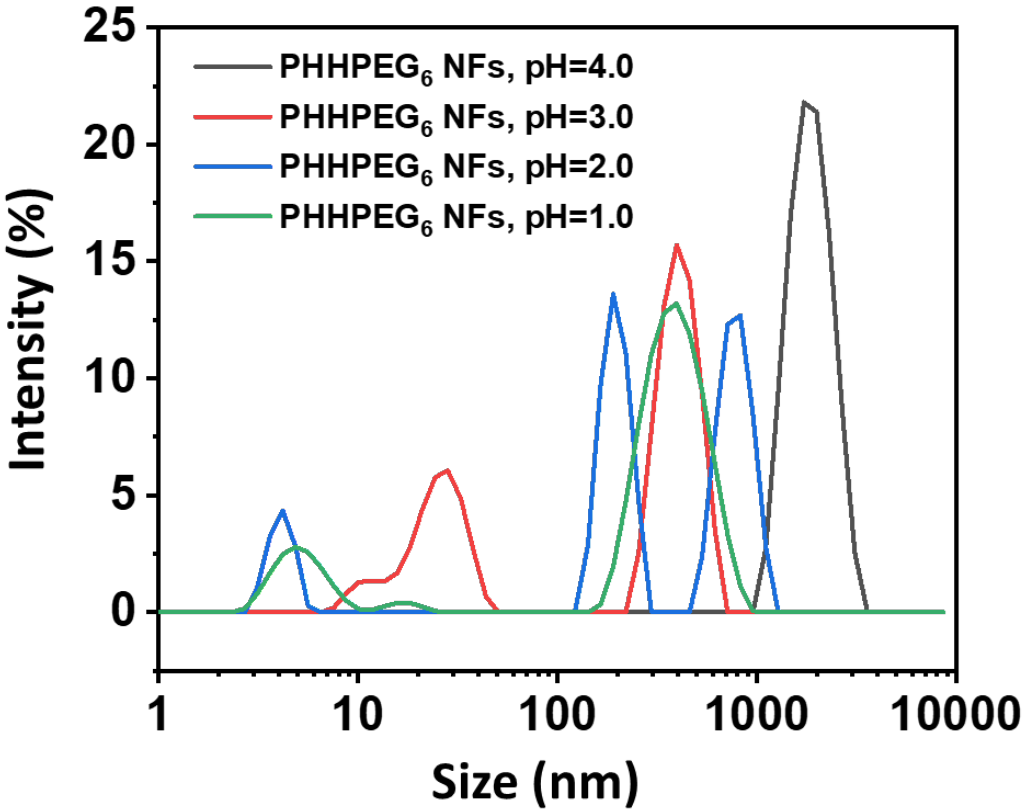

Figure S5. Size distribution of 0.2 mg/mL PHHPEG$_6$ NFs in phosphate buffer solution at pH 4.0, 3.0, 2.0 and 1.0 after 1 h, measured by DLS.

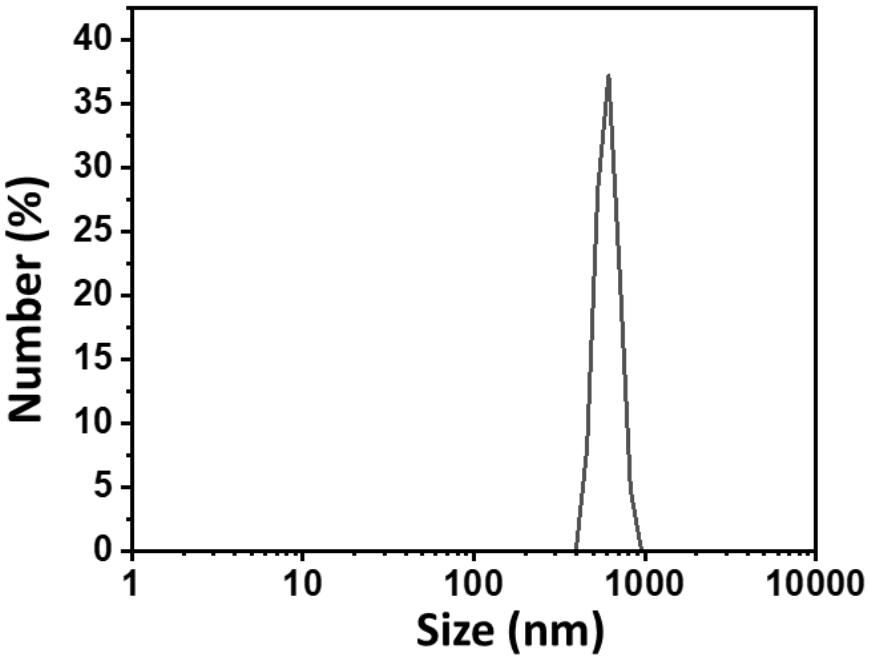

Figure S6. DLS size of PHHPEG$_6$ nanostructures in phosphate buffer solution after adjusting the pH from 5.0 to pH 7.4.

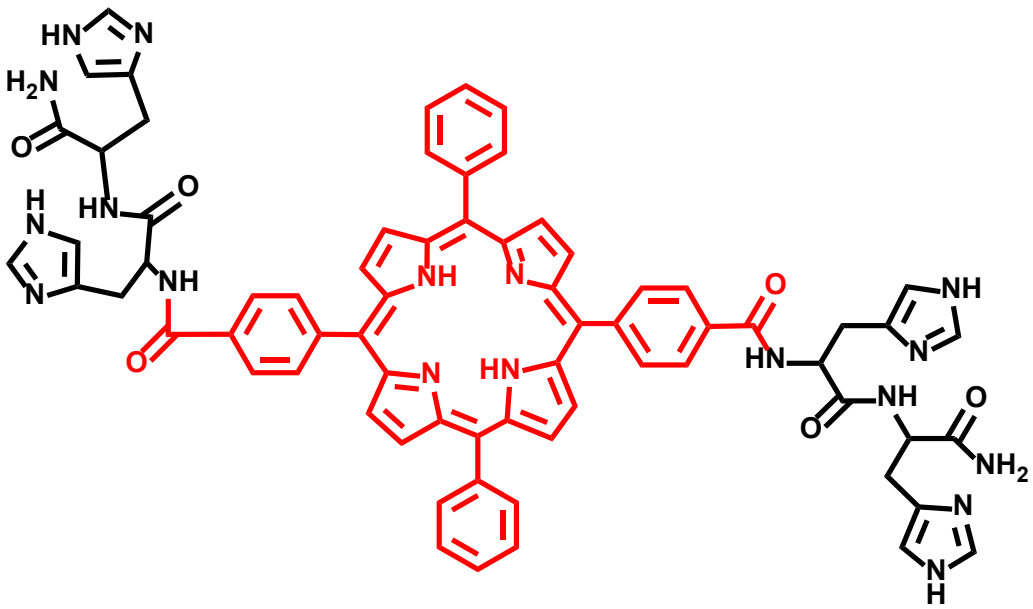

Figure S7. Chemical structure of PHH.

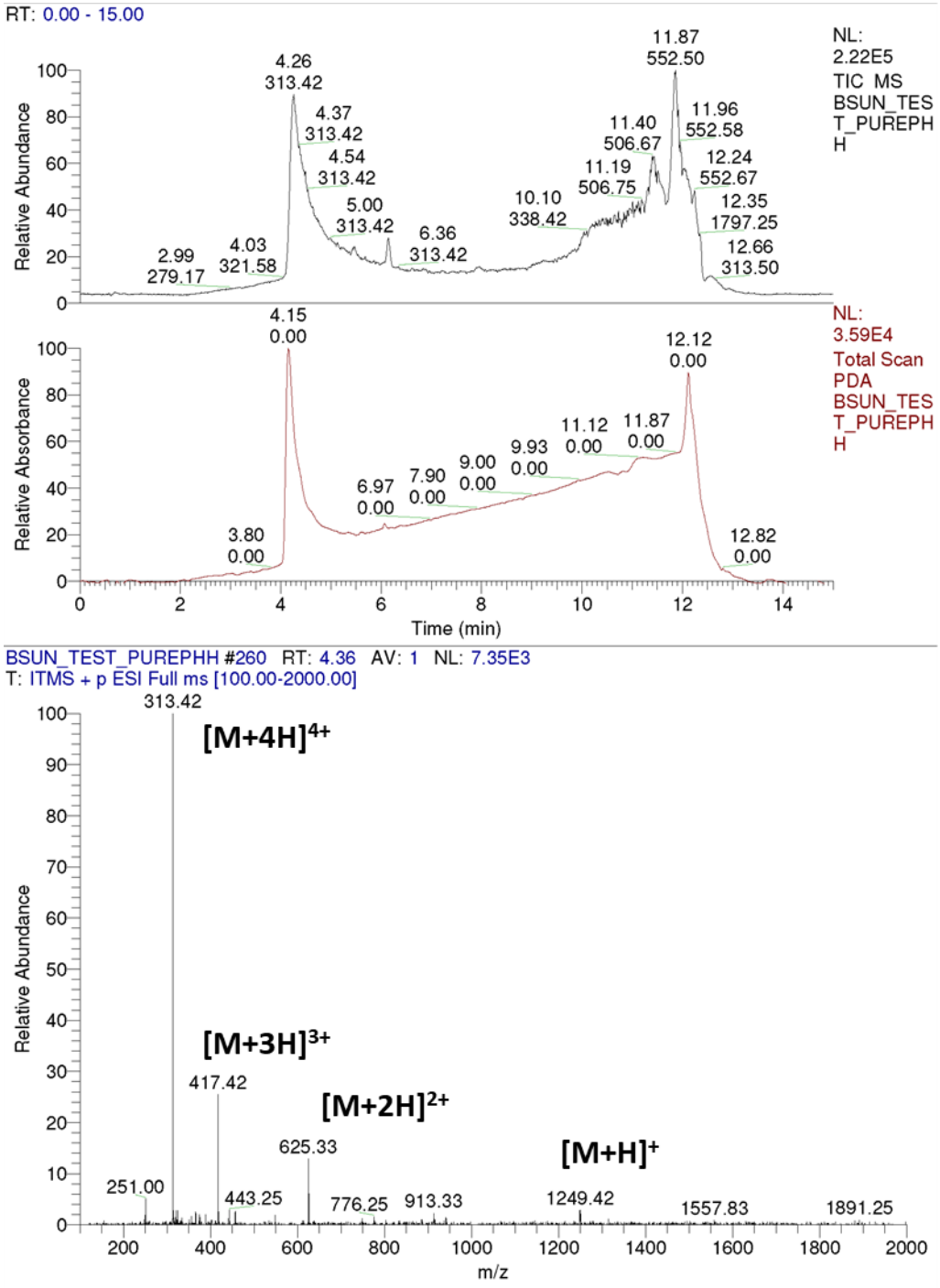

Figure S8. LC-Mass analysis of PHH after purification, RT=4.36; ESI-MS: $[M+H]^+$, $[M+2H]^{2+}$, $[M+3H]^{3+}$, $[M+4H]^{4+}$.

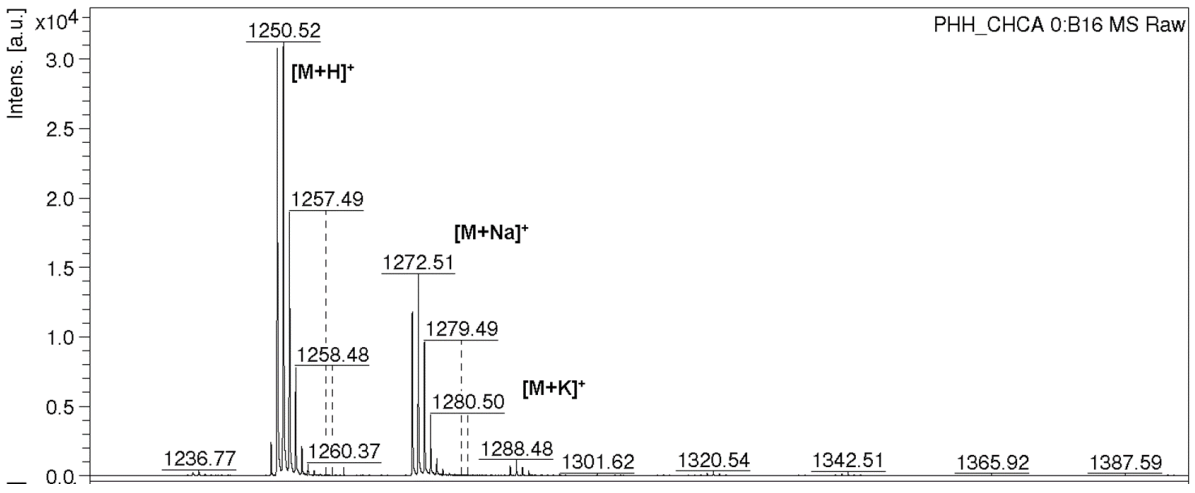

Figure S9. MALDI-TOF Mass analysis of PHH. m/z, [M+H]+, 1250.52, [M+Na]+, 1272.51, [M+K]+, 1288.48.

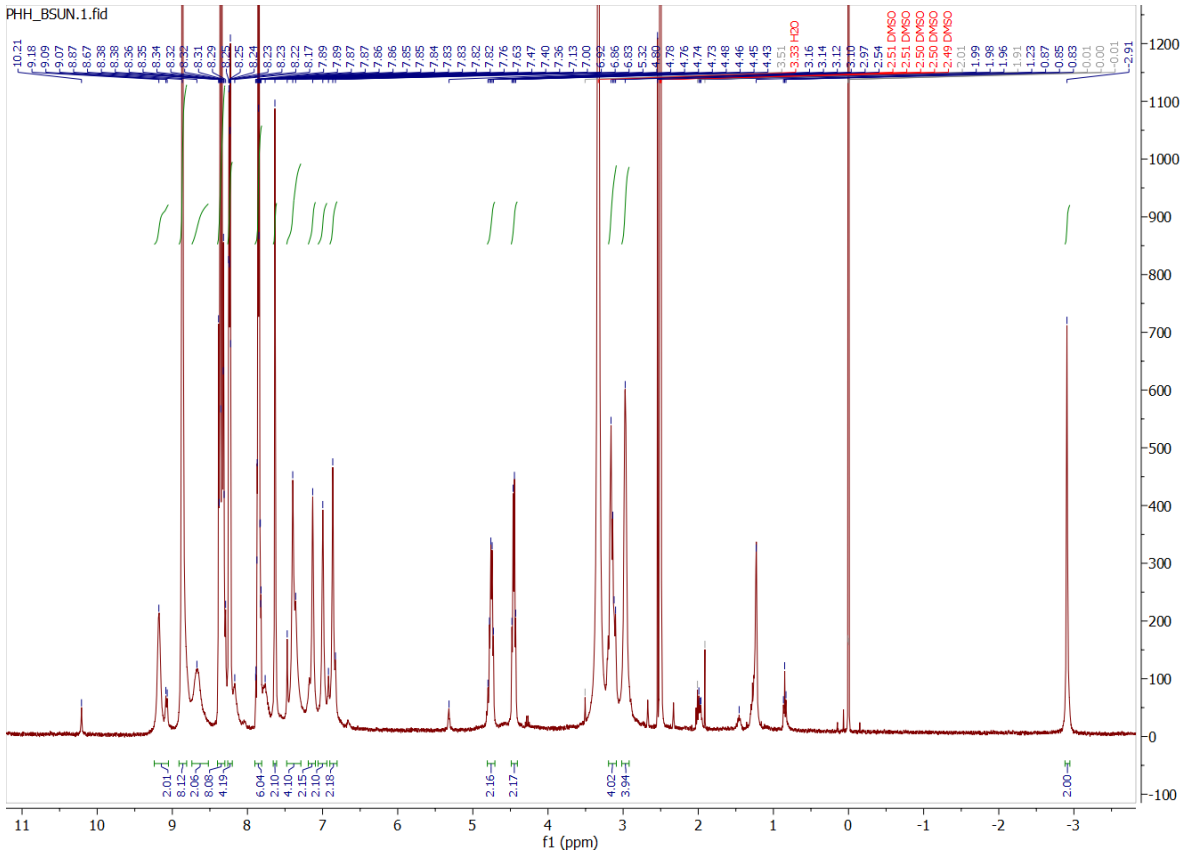

Figure S10. H-NMR of PHH molecules in DMSO-d6.

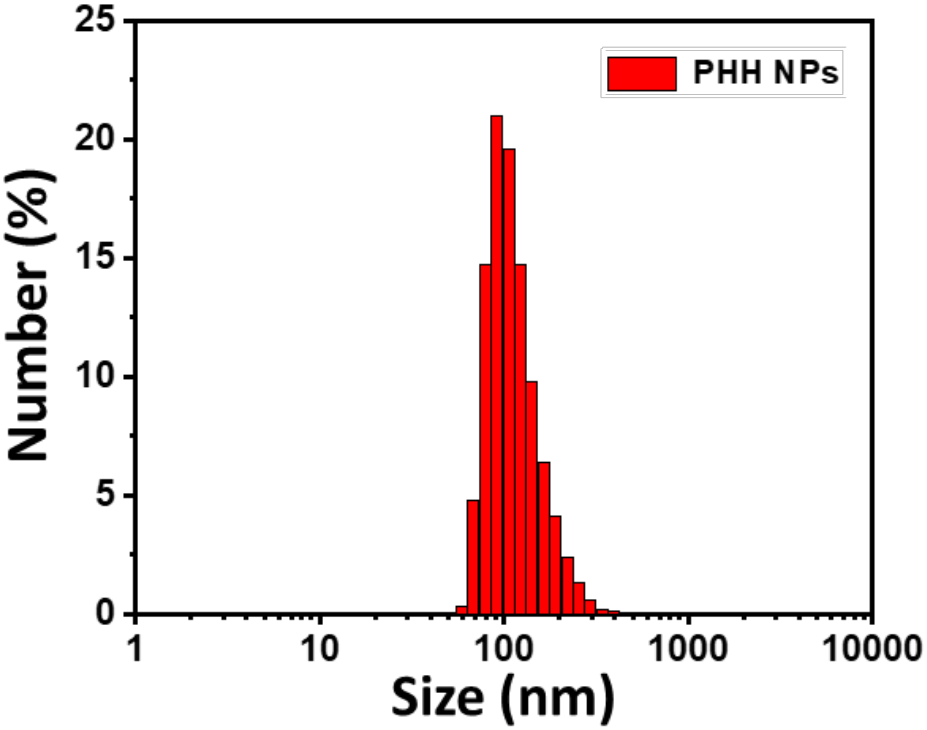

Figure S11. The DLS size distribution of PHH NPs.

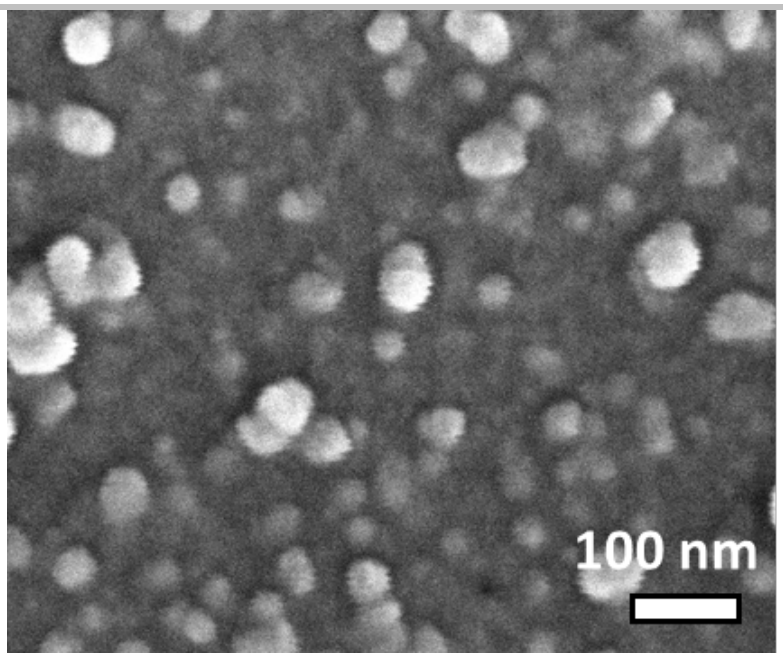

Figure S12. SEM image of PHH NPs.

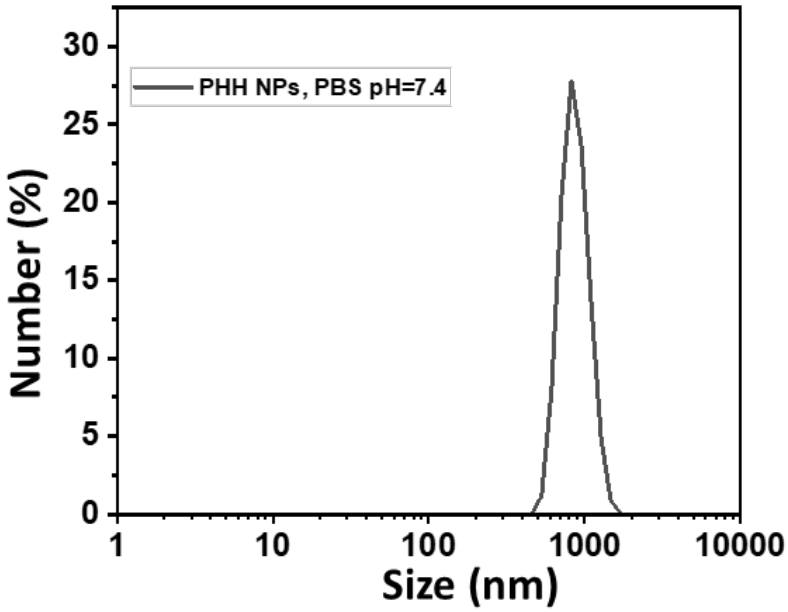

Figure S13. DLS sizes of PHH NPs in phosphate buffer solution at pH 7.4 after 1 h.

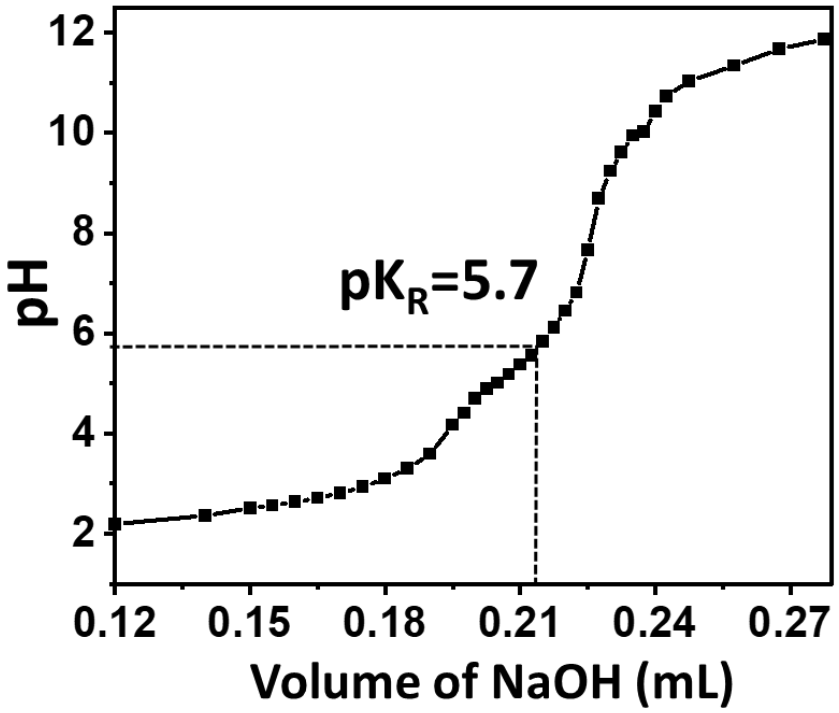

Figure S14. The pH titration curve of PHHPEG$_6$. The pKa of the imidazole group was calculated to be 5.7.

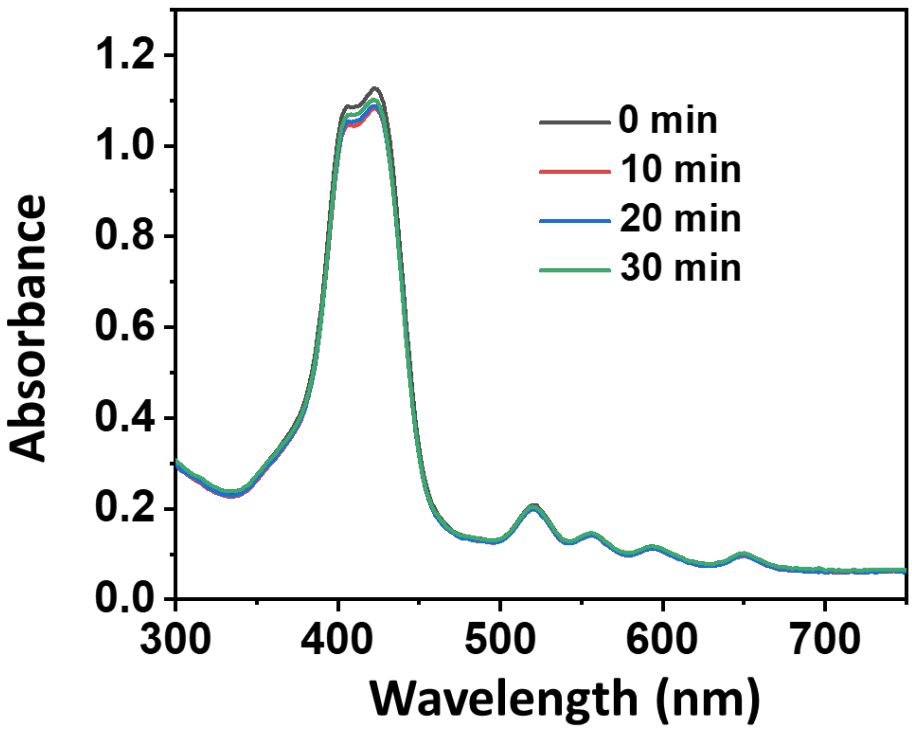

Figure S15. Absorption spectrum of PHHPEG$_6$ NFs monitored at specific time intervals after light irradiation. The light intensity is 100 mW/cm^2.

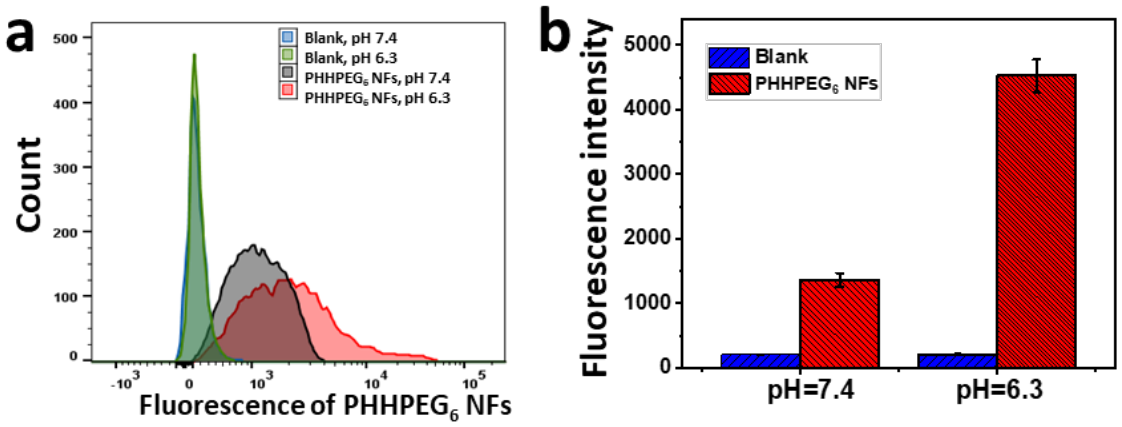

Figure S16. a) Flow cytometry histograms of MCF-7 cells treated with PHHPEG$_6$ NFs and non-treated cells at pH 7.4 and 6.3. b) Mean fluorescence intensities of PHHPEG$_6$ NFs of (a), n=3.

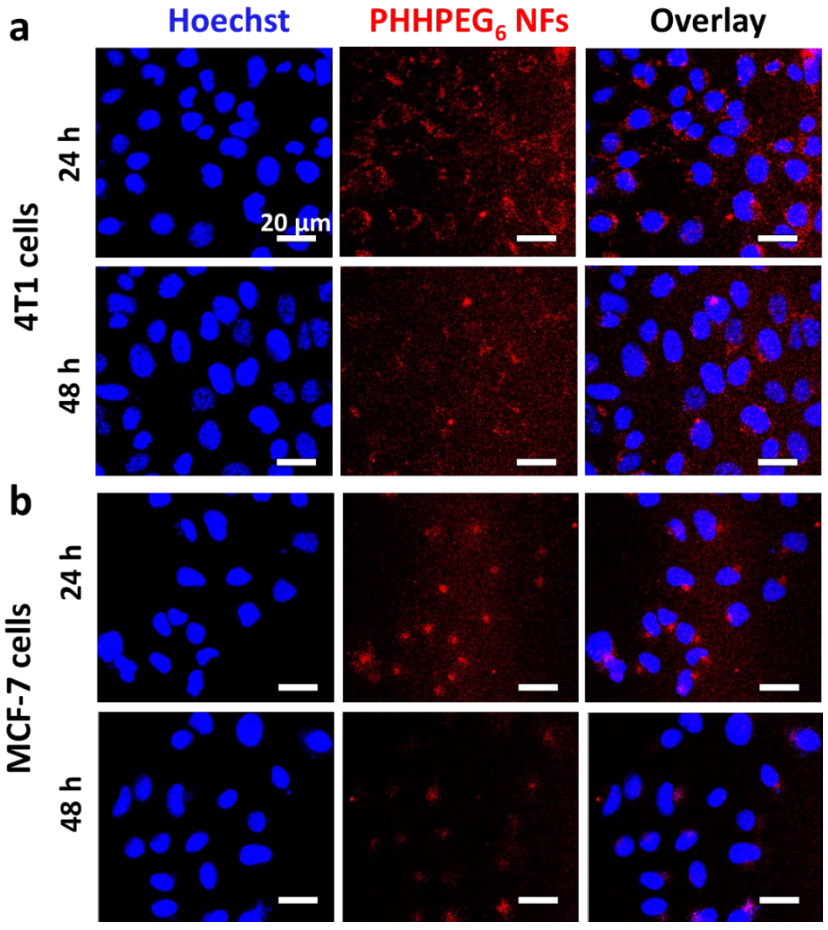

Figure S17. Confocal microscope images of a) 4T1 cells and b) MCF-7 cells co-cultured with 200 µg/mL PHHPEG$_6$ NFs for 24 h at pH 7.4 and 6.3 (upper). After washing NFs from cells and culturing the cells for another 24 h, the fluorescence of the cells after 48 h was measured (bottom).

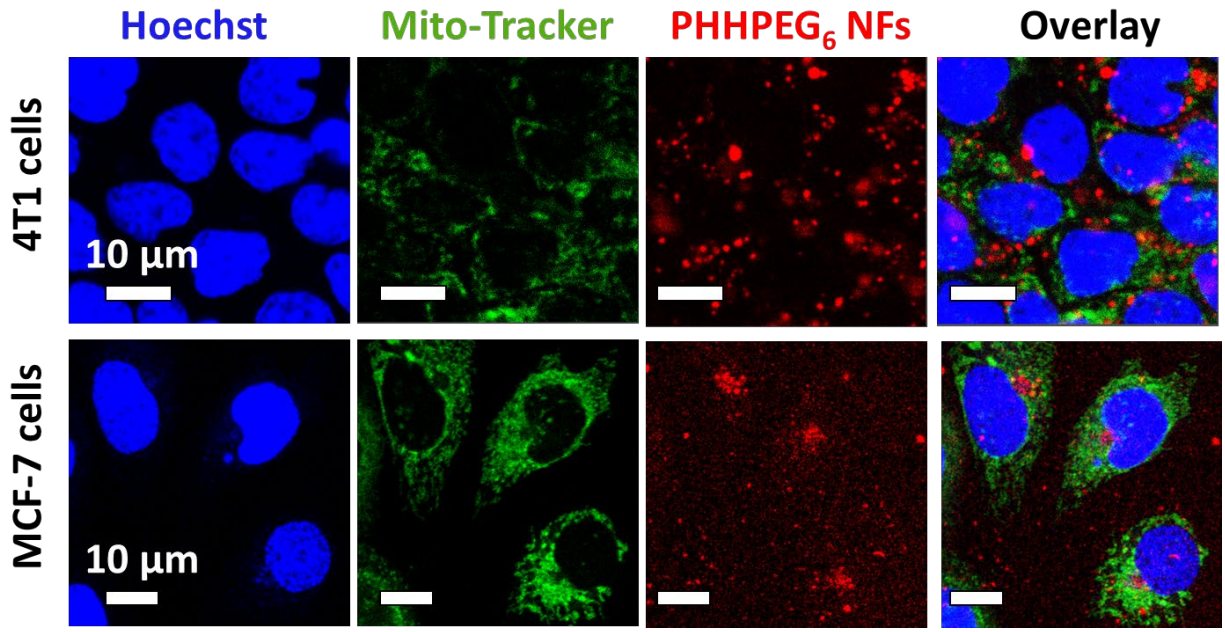

Figure S18. Confocal microscope images of 4T1 cells and MCF-7 cells treated with 200 µg/mL $PHHPEG_6$ NFs for 24 h and stained with Hoechst 33342 and MitoTracker green, respectively.

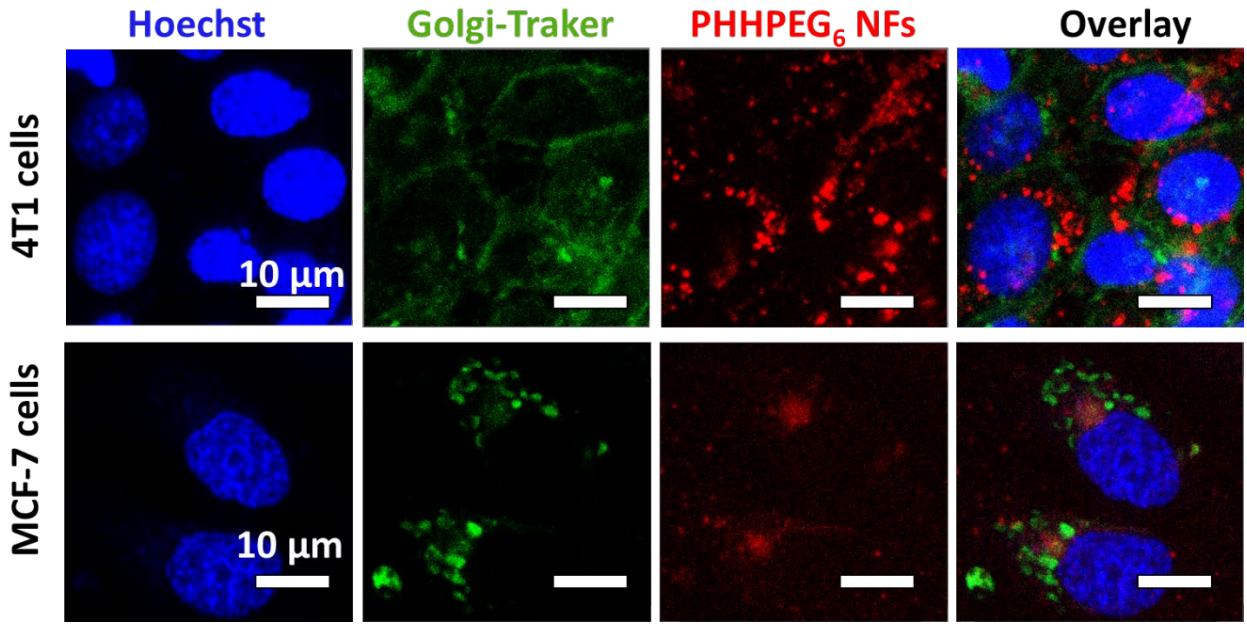

Figure S19. Confocal microscope images of 4T1 cells and MCF-7 cells treated with 200 µg/mL $PHHPEG_6$ NFs for 24 h and stained with Hoechst 33342 and GolgiTracker green, respectively.

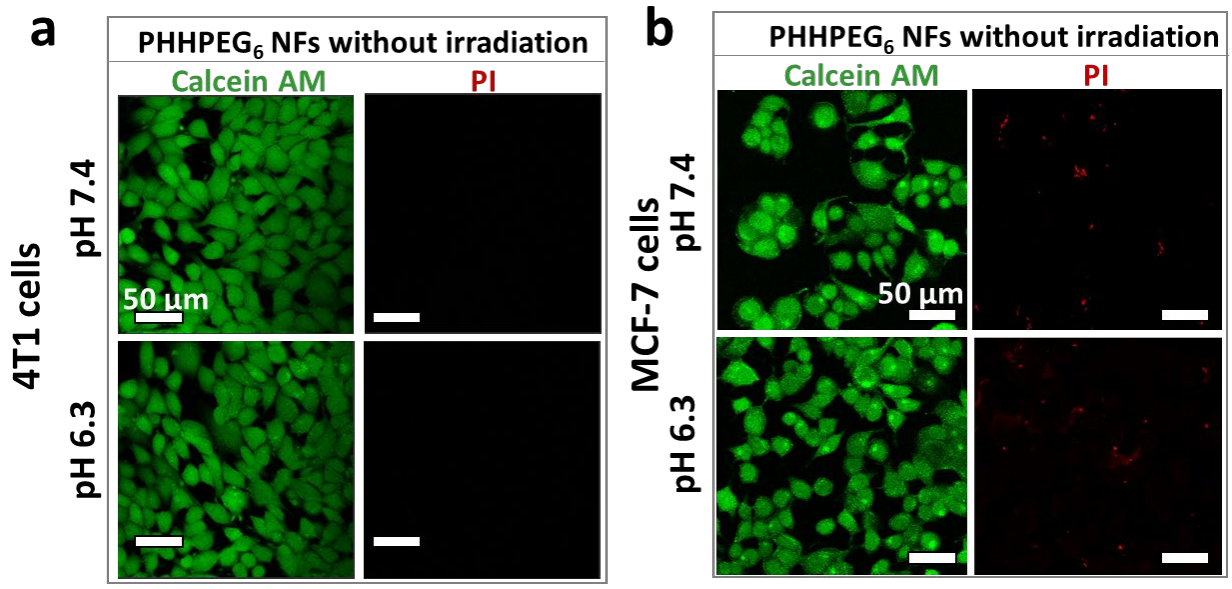

Figure S20. CLSM images of a) 4T1 cells and b) MCF-7 cells stained with calcein-AM/PI and incubated with $PHHPEG_6$ NFs at pH 7.4 and 6.3 without laser irradiation. The concentration of $PHHPEG_6$ NFs used for 4T1 cells was 50 µg/mL, and for MCF-7 cells was 200 µg/mL.

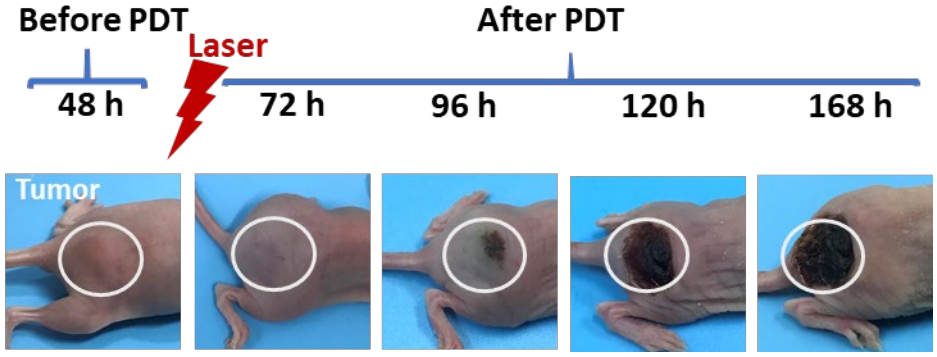

Figure S21. Photographs of mice before PDT at 48 h post injection, and after PDT at 72, 96, 120 and 168 h post injection.

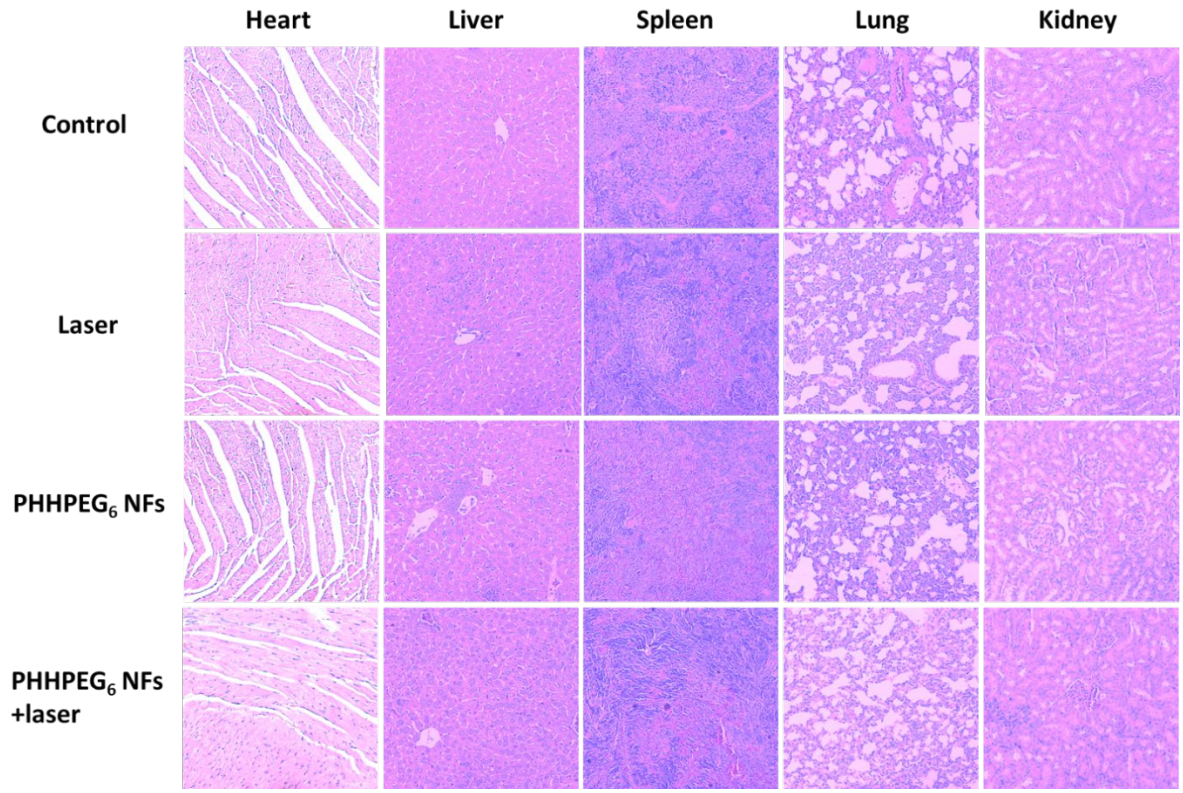

Figure S22. Hematoxylin and eosin (H&E)-stained histological images of tissues harvested at the end of observation.