Supporting Information

Acid-Activatable Transmorphic Peptide-Based Nanomaterials for Photodynamic Therapy

Bingbing Sun, Rui Chang, Shoupeng Cao, Chengqian Yuan, Luyang Zhao, Haowen Yang, Junbai Li, Xuehai Yan,* and Jan C. M. van Hest*

anie_202008708_sm_misellaneous_information.pdf
Table of Contents

1. Materials

2. Synthesis and Methods
   2.1 Peptide-porphyrin synthesis
   2.2 Characterization of PWG molecules
   2.3 Self-assembly of PWG nanoparticles
   2.4 Evaluation of the equivalence point and pKa for PWG
   2.5 Characterization of PWG nanostructures
   2.6 The pH-responsiveness of PWG nanoparticles
   2.7 All-atom molecular dynamics (AAMD) simulations
   2.8 Singlet oxygen generation of PWG nanostructures in solution
   2.9 Heat generation of PWG nanostructures in solution
   2.10 Intracellular trafficking of PWG nanostructures
   2.11 Singlet oxygen generation in vitro
   2.12 Cytotoxicity assay in vitro
   2.13 Tumor model
   2.14 In vivo biodistribution analysis
   2.15 In vivo antitumor efficacy
   2.16 Statistical analysis

3. Supplementary figures and tables
   Figure S1. LC-Mass analysis of PWG after purification, RT=9.88-10.57; ESI-MS: [M+H] +.
   Figure S2. MALDI-TOF Mass analysis of PWG.
   Figure S3. 1H-NMR spectrum of PWG.
   Figure S4. 13C-NMR spectrum of PWG.
   Figure S5. UV-vis absorption of PWG in ethanol and PWG nanoparticles in water at the same concentration of 0.25 mg mL−1.
   Figure S6. Size and PDI of PWG nanoparticles in DMEM containing 10% FBS (pH 7.4) as a function of time measured by DLS.
   Figure S7. PDI of PWG nanoparticles in phosphate buffer solution at pH 7.4, 6.5 and 5.0 after 12 h incubation, measured by DLS.
   Figure S8. Heat generation of PWG nanoparticles at pH 7.4 and nanofibers at pH 6.5 and pH 5.0 when irradiated with a 660 nm laser (2 W cm−2).
   Figure S9. CLSM images of intracellular 1O2 generation in MCF-7 cells treated with PWG nanoparticles at pH 7.4 and 6.5 without illumination by DCF-DA assay.
   Figure S10. Hematoxylin and eosin (H&E)-stained histological images of tissues harvested at the end of observation.
Experimental Procedures

1. Materials
5,15-(di-4-carboxyphenyl)-10,20-(diphenyl)porphyrin (98%) was purchased from PorphyChem, France. Fmoc-Gly-Wang resin was purchased from Rapp Polymere. 9,10-Anthracenediyi-bis(methylene)dimalonic acid (ABDA), 2',7'-dichlorofluorescein diacetate (DCFH-DA), N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), N,N-diisopropylethylamine (DIEPA), N,N-diisopropylcarbodiimide (DIC), Fmoc-Trp(Boc))-OH, Oxyma Pure (ethyl 2-cyano-2-(hydroxyimino)acetate), trisopropylsilane (TIS), trifluoroacetic acid (TFA), dimethylformamide (DMF), dimethylsulfoxide (DMSO), diethylether and all other chemicals were supplied by Sigma-Aldrich. Fmoc-Gly Wang resin was purchased from 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 Peptide-porphyrin synthesis
Peptides were synthesized via Fmoc solid-phase peptide synthesis using an automated Intavis MultiPep RSi peptide synthesizer. Fmoc-Gly Wang resin (0.68 mmol/g loading) was used for the synthesis of the peptide. Fmoc-deprotection was achieved using 20% piperidine in DMF. Fmoc-Trp(Boc)-OH was dissolved in DMF and coupled to the resin using DIPEA/HBTU (2:1) as activators. Then 5,15-(di-4-carboxyphenyl)-10,20-(diphenyl)porphyrin was dissolved in DMF and coupled to the resin using DIC/Oxyma Pure (1:1.5) as activators. After synthesis, resin cleavage of the protected peptide was 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 five times and lyophilized.

The peptides were further purified using a preparative reversed-phase high performance liquid chromatography (HPLC) system which comprised an 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 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⁻¹ and a 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 Na2CO3 and centrifuged to remove residual TFA. Then, the peptides were washed with MilliQ water three times to remove residual Na2CO3.

2.2 Characterization of PWG molecules
1H and 13C 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 x 2.00 mm column with a gradient 5%−100% acetonitrile in H2O 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.

2.3 Evaluation of the equivalence point and pKa for PWG
The equivalence point and pKa value of PWG were obtained by an acid-base titration method. PWG was dissolved in deionized water (1 mg mL⁻¹) and the pH was adjusted to 2 with 1 M HCl. The solution was titrated by the dropwise addition of 0.1 M NaOH solution to obtain the pH profile. The average pH value from triplicate titrations was plotted against the volume of added NaOH solution.

2.4 Self-assembly of PWG nanoparticles
The PWG powder was dissolved in DMSO at a concentration of 25 mg mL⁻¹. Then, 20 µL of PWG solution was diluted into 1 mL MilliQ water under ultrasonication for 30 s to form PWG nanoparticles. The PWG nanoparticles were aged one day before use.

2.5 Characterization of PWG nanostructures
Cryo-TEM experiments were performed using a 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
2.6 The pH-responsiveness of PWG nanoparticles
To investigate the pH-responsiveness of the PWG nanostructures, 0.5 mg mL\(^{-1}\) PWG nanoparticle stock solution was diluted to 0.1 mg mL\(^{-1}\) with 0.1 M phosphate buffer solution at pH 7.4, 6.5 and 5.0, respectively. The size of the PWG nanostructures as a function of time was measured by DLS. The fluorescence spectra (Ex: 518 nm, Em: 600–800 nm) and absorption spectra were recorded by a Spark 10 M microplate reader (TECAN, Switzerland).

2.7 All-atom molecular dynamics (AAMD) simulations
The AAMD simulations were performed using the Gromacs (Version 5.1.4) package. The Amber03 force field was used for amino acid residues and the GAFF force field for the porphyrin moiety. Atomic charges were generated by the RESP method as implemented in the antechamber program. The systems for AAMD simulation consisted of 2 porphyrin-peptide conjugated molecules (PWG and PWG 1) in a cubic water box and neutralized with sodium ions (if necessary). The porphyrins were initially randomly distributed in the water box. The systems were first energy-minimized utilizing the conjugate-gradient algorithm and then equilibrated through running for 500 ps NVT simulations followed by 500 ps NPT simulations. Production runs in the NPT ensemble were then run for 50 ns at 298 K and 1 bar, employing the leapfrog algorithm with a time step of 2 fs to integrate the equations of motion. The electrostatic forces were treated with the particle-mesh Ewald approach. Both the cutoff values of van der Waals forces and electrostatic forces were set to be 1.2 nm. The LINCS algorithm was utilized to preserve bonds.

2.8 Singlet oxygen generation of PWG nanostructures in solution
To examine the influence of buffer pH on the photoactivity of the PWG nanostructures, the PWG nanoparticles formed in water (0.5 mg mL\(^{-1}\)) was diluted to 0.1 mg mL\(^{-1}\) using 0.1 M phosphate buffer solution at pH 7.4, 6.5 and 5.0. After 12 h, the singlet oxygen probe ABDA was added at a final concentration of 100 µM. The PWG nanostructure solution was then illuminated with a 660 nm laser (BeamQ Lasers) at a photodensity of 0.2 W cm\(^{-2}\) for 15 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, 6.5 and 5.0 under light illumination was used as the control.

2.9 Heat generation of PWG nanostructures in solution
200 µL of PWG nanoparticles formed in water (1 mg mL\(^{-1}\)) was diluted to 1 mL (0.2 mg mL\(^{-1}\)) using 0.1 M phosphate buffer solution at pH 7.4, 6.5 and 5.0, respectively. After 12 h, the solution was placed in a quartz cuvette and was irradiated with a 635 nm laser for 10 min. MilliQ water was used as a control group. A thermocouple probe with a digital thermometer was used to measure the temperature in real time.

2.10 Intracellular trafficking of PWG nanostructures
MCF-7 cells were seeded in a µ-Slide 8 Well plate (5 × 10\(^4\) cells/well). After culturing for 24 h, the cells were incubated with PWG nanoparticles (25 µg mL\(^{-1}\)) at pH 7.4 for 24 h, and then were washed three times using PBS. Subsequently, the cells were stained with LysoTracker Green (100 nM) (Life Technologies) for 30 min and Hoechst 33342 (10 µg mL\(^{-1}\)) (Life Technologies) for 10 min at 37 °C. Then, the cells were washed three times with PBS, resuspended in 200 µL of medium, and immediately were observed using CLSM.

2.11 Singlet oxygen generation in vitro
MCF-7 cells were seeded in a µ-Slide 8 Well plate (5 × 10\(^4\) cells/well). After culturing for 24 h, the cells were incubated with PWG nanoparticles (25 µg mL\(^{-1}\)) at pH 7.4 and 6.5 for 12 h. After that, the cells were washed three times with PBS. The cells were incubated with the fluorescent probe DCBF-DA (20 µM) for 30 min and Hoechst 33342 (10 µg mL\(^{-1}\)) for 10 min at 37 °C in the dark, and then were washed three times with PBS, followed by resuspension in 200 µL of PBS. The cells were subsequently treated with a 660 nm laser for 10 min at a photodensity of 0.12 W cm\(^{-2}\) and were successively examined using CLSM.

2.12 Cytotoxicity assay in vitro
MCF-7 cells were seeded in 96-well tissue culture plates (5 × 10\(^3\) cells/well). After culturing for 24 h, the cells were incubated with PWG nanoparticles at different concentrations (0, 5, 10, 15, 20, 25 µg mL\(^{-1}\)) at pH 7.4 and 6.5. After incubation for 12 h, the cells were washed three times with PBS and resuspended in 100 µL of DMEM. The cells were subsequently treated with a 660 nm laser for 10 min at a photodensity of 0.12 W cm\(^{-2}\) and were subsequently cultured for an additional 24 h. The cell viability was then analyzed by the MTT assay. MCF-7 cells treated with PWG nanoparticles without light illumination were set as the control.

2.13 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 Institutional Animal Care and Use Committee in compliance with Chinese law for experimental animals with an approval number of IPEAECA2019106. Female BALB/c-nu mice were purchased from Beijing Weitong Lihua Experimental Animal Technical Co., Ltd. and were housed in an environmentally controlled animal facility with a regular 12/12 light/dark cycle. MCF-7 cells were collected and suspended in PBS at a concentration of $6 \times 10^7$ cells mL$^{-1}$. Each mouse was injected with a 100 µL suspension solution of MCF-7 cells on the hind hip. The in vivo experiments were carried out when the tumors reached a size of approximately 100 mm$^3$.

2.14 In vivo biodistribution analysis
200 µL of 0.2 mg mL$^{-1}$ PWG nanoparticles in an aqueous 5% glucose solution were injected into MCF-7 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 time points as 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, 144 h, 168 h, 240 h, 360 h by observing the fluorescence of PWG. At 24, 72 and 360 h post injection, the mice were sacrificed, and the harvested organs (liver, spleen, kidneys and heart) and tumors were imaged by this imaging system. The fluorescence intensity was obtained using the built-in software for the imaging system.

2.15 In vivo antitumor efficacy
Twenty nude mice implanted with MCF-7 tumors were randomly divided into four groups ($n = 5$). When the tumor volume reached 100 mm$^3$, the mice in each group were injected with 200 µL of 0.2 mg mL$^{-1}$ PWG nanoparticles (group 1, 1#), 5% glucose solution (control group, 2#), 0.2 mg mL$^{-1}$ PWG nanoparticles (group 3, 3#), and 5% glucose solution (group 4, 4#) respectively via the tail vein. At 24 h post injection, the tumor sites of the groups 1# and 4# were irradiated by a 660 nm laser (0.3 W cm$^{-2}$) for 20 min. In the following 21 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 21$^{st}$ day post treatment and the major organs (heart, liver, spleen, lung, and kidneys) were harvested and fixed with a 4 wt% paraformaldehyde solution for histological analysis to evaluate the biosafety of the PWG nanostructures. After embedded in paraffin and stained by hematoxylin and eosin (H&E), the organs were examined by hematoxylin-eosin (H&E) staining.

2.16 Statistical analysis
Data are expressed as mean ± standard deviation.

3. Supplementary figures and tables
Figure S1. LC-Mass analysis of PWG after purification, RT=9.88-10.57; ESI-MS: [M+H] +.

Figure S2. MALDI-TOF Mass analysis of PWG. m/z, [M+H] +, 1189.44.
Figure S3. $^1$H-NMR spectrum of PWG.

$^1$H NMR (600 MHz, DMSO-d6, Figure S3) $\delta$ = 10.86-10.85 (d, 2H), 9.14-9.12 (d, 2H), 8.82-8.81 (d, 8H), 8.30-8.25 (q, 8H), 8.22 - 8.20 (m, 4H), 7.85 – 7.81 (m, 6H), 7.74 - 7.72 (d, 2H), 7.64 - 7.62 (t, 3H), 7.37 - 7.33 (m, 4H), 7.11-7.01 (m, 4H), 4.92 - 4.86 (m, 2H), 3.45 – 3.38 (m, 7H), 3.28 – 3.24 (m, 4H), -2.93 (s, 2H).

Figure S4. $^{13}$C-NMR spectrum of PWG.

$^{13}$C NMR (600 MHz, DMSO-d6): $\delta$ = 170.83, 170.53, 166.73, 144.57, 136.66, 134.64, 134.51, 134.16, 127.85, 127.48, 126.53, 123.92, 121.36, 120.67, 119.67, 118.75, 111.85, 111.69, 55.31, 44.76, 27.61 ppm.
Figure S5. UV-vis absorption of PWG in ethanol and PWG nanoparticles in water at the same concentration of 0.25 mg mL⁻¹.

Figure S6. Size and PDI of PWG nanoparticles in DMEM containing 10% FBS (pH 7.4) as a function of time measured by DLS.
Figure S7. PDI of PWG nanoparticles in phosphate buffer solution at pH 7.4, 6.5 and 5.0 after 12 h incubation, measured by DLS.

Figure S8. Heat generation of PWG nanoparticles at pH 7.4 and nanofibers at pH 6.5 and pH 5.0 when irradiated with a 660 nm laser (2 W cm\(^{-2}\)). The laser was switched off after irradiation for 10 min.
Figure S9. CLSM images of intracellular $^{1}$O$_{2}$ generation in MCF-7 cells treated with PWG nanoparticles at pH 7.4 and 6.5 without illumination by DCF-DA assay.

Figure S10. Hematoxylin and eosin (H&E)-stained histological images of tissues harvested at the end of observation.
Author Contributions

J.H. conceived the project. J.H., B.S., X.Y., S.C. and R.C. designed the experiments. B.S., R.C., and L.Z. performed all the experiments. B.S., J.H., X.Y., J.L., R.C., C.Y., and H. Y. analyzed the data and composed the manuscript. All authors discussed the results and commented on the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.