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Electronic Supplementary Information

Facile Self-assembly of Porphyin-embedded Polymeric Vesicles for Theranostic Applications

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1. Preparation and characterization of PPLA porphysomes without or with air loading

PPLA porphysomes were prepared using an oil-in-water solvent evaporation method. Briefly, PPLA conjugate powder (2 mg) was dissolved in acetone (1 mL) and subsequently added to d.d. water (5 mL) while stirring at 450 rpm for 12 hours to evaporate the organic solvent. Similarly, the nanoparticles were loaded with air by pumping air into the d.d. water while stirring at 450 rpm while the polymer solution was added; this process continued until all of the polymer solution was depleted. Then, the organic solvent was removed by continuously pumping air into the sample for at least 30 minutes. The size distribution and polydispersity of the nanoparticles were measured using DLS (Nano ZS90, Malvern Instruments Ltd, Worcestershire, UK). The aqueous solution of PPLA porphysomes was evaluated at 25 ℃ at a fixed angle of 90° for the DLS measurement. The morphology of the nanoparticles was examined using transmission electron microscopy (TEM; JEOL JEM-1400 cryo-TEM, JEOL, Ltd., Akishima, Japan). The detailed structure of the nanoparticles was examined using SANS (CG2-SANS; Oak Ridge, Tennessee, USA). Details of the SANS experiments and the curve fitting models are described in the next section.

2. Fluorescence spectra of PPLA porphysomes in various pH solutions

The effect of pH on the PPLA porphysomes was also studied using buffer solutions with varying pH values, from 4.05 to 9.93. The fluorescence intensity of the PPLA porphysomes was not significantly altered under different pH conditions. Therefore, the PPLA porphysomes produced in this study were highly stable with respect to particle size and their fluorescence properties, which is important for retaining their PDT efficacy after long-term storage at room temperature.

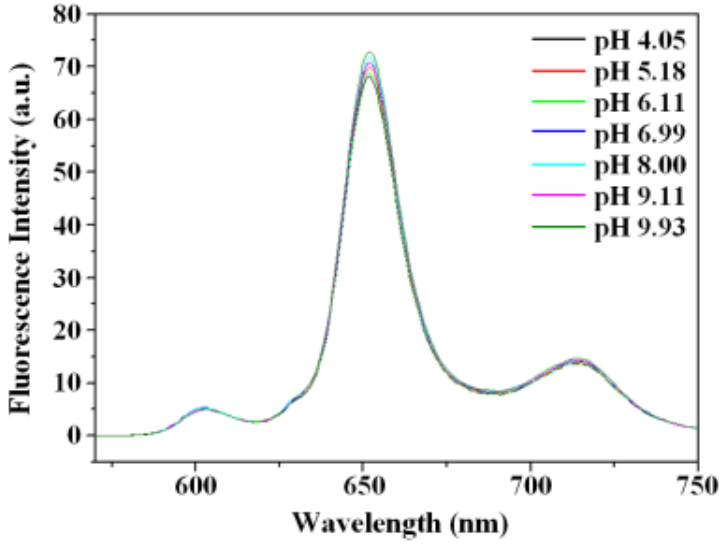

Fig. S1: Fluorescence spectra of PPLA porphysomes in various pH solutions.

3. Singlet oxygen production of PPLA porphysomes-mediated PDT

To measure the amount of $^1O_2$ produced by the PPLA porphysomes, the Singlet Oxygen Sensor Green (Molecular Probes Inc., Leiden, The Netherlands) sensing probe was used, which is highly selective probe for detecting $^1O_2$ without presenting an appreciable response to hydroxyl radicals or superoxides. In brief, 1 μM of Singlet Oxygen Sensor Green and 0.2 mg/mL samples (PPLA conjugate dropped into water directly or PPLA porphysomes) were first dissolved in 100 mM pH 7.5 tris buffer and the fluorescence intensity was determined using a SpectraMax M2^e multi-detection microplate reader. After 30 seconds, the samples were exposed to light radiation to stimulate the photochemical reaction, and the fluorescence intensity was measured again using a SpectraMax M2^e multi-detection microplate reader. In the presence of $^1O_2$, the sensing probe emits a green fluorescence similar to that of fluorescein (Ex/Em = 488/524 nm) that can be identified using fluorescence spectroscopy.

The light source for activating PPLA porphysomes consisted of a bank of four fluorescent tubes (Osram 18W/67) emitting in the spectral region 390-450 nm with the highest intensity at approximately 420 nm. The total fluence rate was 7 mW/cm^2.

4. Characterization of PPLA porphysomes by Small Angle Neutron Scattering (SANS)

The same PPLA porphysomes preparation procedure was employed in D_2O rather than water to form a 0.2 wt% of PPLA porphysome solution for the SANS measurement. The 0.2 wt% solution was then diluted with D_2O to produce the final solutions with polymer concentrations of 0.05 and 0.1 wt%. The SANS experiments were performed on the CG2-SANS instrument located at the High Flux Isotope Reactor (HFIR) at Oak Ridge National Laboratory (Oak Ridge, Tennessee, USA). Neutrons with a wavelength of 4.75 Å and two sample-to-detector distances (s.d.d.) of 4 m and 18.5 m were used to cover a q range from 0.004 Å^{-1} to 0.26 Å^{-1}. All of the 2-D raw data were properly corrected for the sample and empty cell transmissions and for the background scattering, and then the data were reduced to 1-D intensity plots as a function of q.

5. SANS curve fitting model

The reduced scattering intensity, $I(q)$, in the SANS experiment can be expressed as a product of the form factor, $P(q)$, which contains information about the shape and dimensions of the scattering particles, and the structure factor, $S(q)$, which describes the interparticle interactions:

$$I(q) = \psi\,(\Delta\rho)^2\,P(q)\,S(q)$$

where ψ is the volume fraction of the nanoparticles and $\Delta\rho$ is the contrast of the

scattering length density between the nanoparticles and the solvent.[1] In the dilute region, the interparticle interactions can be neglected; therefore, the analysis of the scattering intensity $I(q)$ can provide the morphology of the particles in the scattering volume.[2]

As mentioned previously, the two possible structures were spherical micelles and vesicles. Both structures have a core-shell type structure and can be described using the same model. The core/shell ratio, i.e., R_c/R_s, was assumed to be a constant, where R_c and R_s are the core and shell radii, respectively. The corresponding form factor can be written as

$$P(q) = \frac{A}{V_s}\left[\frac{3V_c(\rho_c - \rho_s)\,j_1(qr_c)}{qr_c} + \frac{3V_s(\rho_s - \rho_{solv})\,j_1(qr_s)}{qr_s}\right]^2 + bkg$$

where $j_1(qr_i)$ is the first-order spherical Bessel function,

$$j_1(qr_i) = \frac{\sin(qr_i) - qr_c\cos(qr_i)}{(qr_i)^2}$$

and $V_i=(4\pi/3)r_i^3$. Here A, ρ and r express the scale factor, scattering length density and radius, respectively, and the subscripts *s*, *c* and *solv* refer to the shell, core and solvent, respectively.[2-3] The R_c was also assumed to follow a Schulz distribution, $f(Rc)$, as follows.

$$f(R) = \frac{p^{-2/p^2}}{\langle R_o\rangle\Gamma(1/p^2)}\left(\frac{r}{\langle R_o\rangle}\right)^{1-p^2/p^2}\exp\left(-\frac{r}{p^2\langle R_o\rangle}\right)$$

where $\langle R_o \rangle$ is the average core radius of the particle, $\Gamma(1/p^2)$ is the Gamma function and p is the polydispersity in the range of 0-1. Polydispersity p also refers to σ/R_o, where σ^2 is the variance of the distribution and R_o is the total particle radius.[4]

In the case of the spherical micelles, the core and shell are porphyrin and PLA, respectively, whereas the vesicle model assumes a D_2O core and a PPLA shell.[5] All of the scattering length densities (core, shell and solvent) were fixed at values that correspond to their physical meaning. Note that the calculated intensities for the micelle and vesicle were normalised by the total spherical volume and shell volume, respectively. During fitting, the calculated intensity was smeared by the instrument resolution function. Each SANS spectra was best fit using the program developed at NIST Center for Neutron Research, which was written in the IGOR PRO 6.0 software package.[6]

6. Characterization of PPLA porphysomes by Static Light Scattering (SLS)

To characterise the restructuring of the PPLA porphysomes, static light scattering (SLS, BI-200SM, Brookhaven Instruments, Co., New York, USA) was utilised, and

intensity traces were obtained at different angles to derive the radius of gyration and the molecular mass of the polymer complex. For the SLS measurement, the incident laser beam has a wavelength at 632.7 nm and the scattered intensities of the dilute PPLA porphysome solutions (0.015-0.05 mg/mL) were collected as a function of the scattering angles. A Zimm plot was constructed to provide the apparent weight average molecular mass ($M_{w,app}$) of the PPLA porphysomes. The SLS measurements were performed at 25℃ in d.d. water at angles between 40° and 140°.

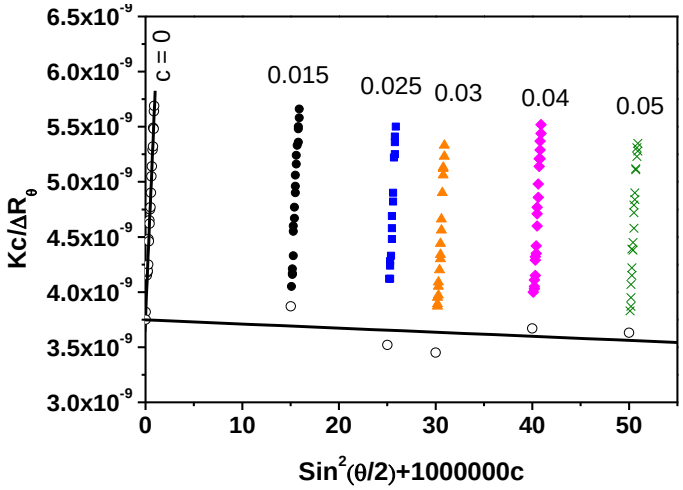

Fig. S2: Zimm plot of PPLA porphysomes was constructed by SLS with various concentrations and various angles.

7. Determination radius of gyration from spherical geometric radii

The radius of gyration (R_g) can be calculated by the following equation: [7]

$$R_g^2 = \frac{3}{5} r_o^2 \frac{1-\left(\frac{r_i}{r_o}\right)^5}{1-\left(\frac{r_i}{r_o}\right)^3} \qquad (1)$$

where r_o and r_i are the outer radius and the inner radius of a sphere, respectively. For the case of infinitely thin shell of sphere ($r_i/r_o \rightarrow 1$), the R_g directly equal to r_o. Similarly, the $R_g^2 = 0.6\ r_o^2$ in the case of solid sphere ($r_i = 0$). In our case, r_i and r_o were measured by SANS (35 nm and 54 nm respectively), then therefore the R_g is calculated to be 46.1 nm.

8. Cell culture and incubation conditions

Human cervical epithelioid carcinoma (HeLa) cells were grown in a 75T culture flask and maintained in a humidified 5% CO_2 incubator at 37 ℃ in DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% foetal bovine serum (FBS) (Gibco

BRL, Gaithersburg, MD) and 1% antibiotics (Antibiotic-Antimycotic, Gibco BRL, Gaithersburg, MD, USA). The cells were subcultured 2~3 times per week using 0.25% trypsin-EDTA.

9. Intracellular distribution of PPLA porphysomes by confocal microscopy

To evaluate the intracellular distribution of PPLA porphysomes, 10^4 HeLa cells were seeded onto glass coverslips with 1 mL of cultured medium in a 35-mm dish and cultured at 35 ℃ with 5% of CO_2 for 24 h. porphyrins (1.4 μM) of the PPLA porphysomes was added to the cells and then cultured for an additional 24 h. After washing three times with PBS, the glass coverslips were sealed onto slides then assessed immediately using Leica-SP5 confocal laser scanning microscope. The porphyrin fluorescence was observed with Ex/Em = 405/600-700 nm.

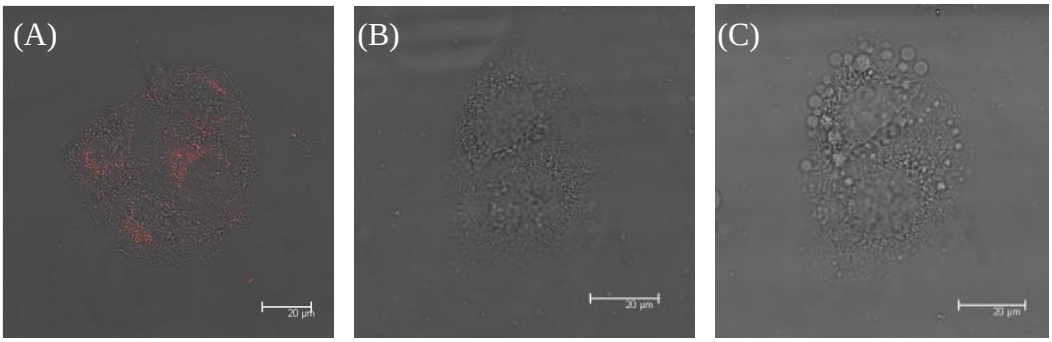

Fig. S3: Intracellular distribution (A) and PDT effects of PPLA porphysomes (1.4 μM) in HeLa cells before (B) and after (C) irradiation (2.1 J/cm^2).

10. Cellular uptake study

For the cellular uptake study, HeLa cells were seeded on 96-well culture plates at a concentration of 5000 cells per well in a 100 μL culture medium. After growing overnight, the cells were exposed to various concentrations (0.005~2.8 μM) of PPLA porphysomes in medium to probe the dose-dependent PPLA accumulation after 24 h. Afterwards, the cells were washed twice with PBS, dissolved in DMSO and the fluorescence intensity of cellular uptake was measured. The cellular uptake of PPLA porphysomes was measured by determining the fluorescence emission of the accumulated porphyrins with a SpectraMax M2^e multi-detection microplate reader (Molecular Devices, Sunnyvale, CA) using an excitation/emission ratio = 420/652 nm.

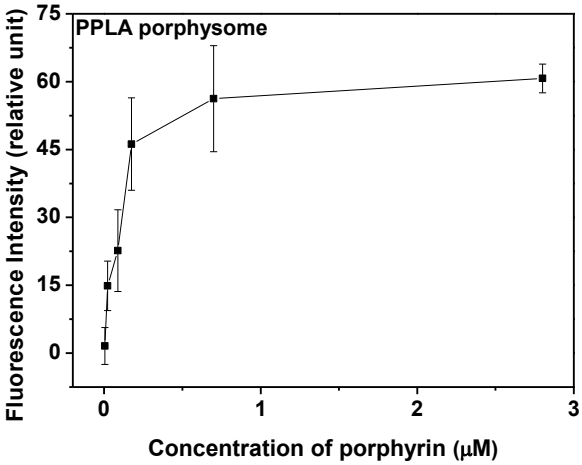

Fig. S4: Cellular uptake of PPLA porphysomes with various concentrations for 24 hrs in HeLa cells.

11. Cytotoxicity and phototoxicity of PPLA porphysomes

To investigate the cytotoxicities of the PPLA porphysomes, HeLa cells were first seeded into 96-well plates at a density of 6000 cells per well and cultured for 24 h. The PPLA porphysomes were then added in two-fold serial dilutions from 2.8 μM to 0.18 μM (porphyrin concentration) in a total volume of 0.1 mL at 37 ˚C for 24 h. The cells were then washed with PBS and immediately exposed to different light doses (0.7 J/cm^2 - 2.1 J/cm^2). After light exposure, the cells were incubated in the dark for 48 h and then evaluated using the MTT assay.[8] The results were read using a scanning multiwell ELISA reader (SpectraMax M2^e, Molecular Devices, Sunnyvale, CA, USA). After the addition of the PPLA porphysomes, all of the procedures were performed under subdued light. The light source for activating the PPLA porphysomes was supplied by a PCI Biotech (LumiSource®, Oslo, Norway) and consisted of a bank of 4 fluorescent tubes (Osram 18W/67) emitting in the spectral region of 390-450 nm with the highest intensity at approximately 420 nm. The total fluence rate was 7 mW/cm^2.

Table S1. Summary of survival fraction of PPLA porphysomes-mediated PDT in HeLa cell line.

Conc. of porphyrin	Survival Fraction of HeLa (%) (N=5)			
	Dark	0.7 J/cm^2	1.4 J/cm^2	2.1 J/cm^2
0.18 μM	1.07±0.03	1.04±0.03	0.97±0.02*	0.92±0.05*
0.35 μM	1.04±0.03	0.99±0.02	0.93±0.06*	0.91±0.07*
0.7 μM	1.07±0.05	0.96±0.04*	0.92±0.08*	0.84±0.04†
1.4 μM	1.07±0.05	0.89±0.04†	0.74±0.12†	0.63±0.05†
2.8 μM	0.97±0.11	0.43±0.04†	0.31±0.11†	0.21±0.06†

*p-value <0.05 and †p-value <0.01 indicated the significance of cell survival fraction in light intensity 0.7 J/cm^2, 1.4 J/cm^2, 2.1 J/cm^2 as comparing with dark in HeLa cells.

12. Ultrasonic imaging of air loading PPLA porphysomes

Ultrasonic imaging was conducted using a Visualsonics Vevo 770® system (VisualSonics Inc., Toronto, Ontario, Canada) operating at a scanning frequency of 40 Hz. Briefly, three layers of 1% agar plates were stacked perpendicularly and immersed in deionised water. To ensure that the holes (diameter: ~8 mm, height: ~10 mm) in the middle layer of agar plate were fully filled with deionised water, the three layers of agar plates were fixed in place using toothpicks. After the plates were fixed, 100 μL of air loaded PPLA porphysomes or deionised water as a control group were injected into the holes in the middle layer of the agar plate using a syringe. Making the transducer closed enough to the top layer of plate and controlled the transducer horizontal movement by Vevo imaging station (VisualSonics Inc., Toronto, Ontario, Canada) with a square scanning are of 1 cm^2 above the holes.

13. Statistical analysis

In a general analysis, the data were expressed as a mean with standard deviation (SD) for continuous variables. A non-parametric method, Kruskal-Wallis test, was performed to compare the cell survival fraction under four conditions, dark, 0.7 J/cm^2, 1.4 J/cm^2, and 2.1 J/cm^2, with a Mann-Whitney U test in a post-hoc comparison between dark vs. and 0.7 J/cm^2, dark vs. and 1.4 J/cm^2, and dark vs. and 2.1 J/cm^2, respectively. All of the statistical analyses were performed using the SPSS 15.0 statistics software package (SPSS Inc., Chicago, IL, USA).

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