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# **Small** Micro

# Supporting Information

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Design of Hydrated Porphyrin-Phospholipid Bilayers with Enhanced Magnetic Resonance Contrast

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## **Supporting Information**

#### Materials

Materials were obtained from Sigma unless otherwise noted. Lipids including DSPE-PEG-2K (1,2distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] and DMPC (1,2dimyristoyl-sn-glycero-3-phosphocholine) were from Avanti Polar Lipids.

#### Methods

#### Generation of PoP and PoP liposomes

Boc-N-HPPH was synthesized from pyropheophorbide-*a* and purified as previous described.<sup>[1]</sup> 4 mL 30% HBr/HOAc was added to 200 mg pyrophrophorbide-a in a round bottom flask. After 2 hr stirring at room temperature, the solvent was removed under high vacuum. The residue was dissolved in 10 mL dry methylene chloride. 400 mg 6-(tert-Butoxycarbonylamino)-1-hexanol and 300 mg potassium carbonate was added. The reaction mixture was stirred at room temperature for 2 hr under argon and poured into 100 mL water. 3 x 100 mL methylene chloride was used to extract and collect the product. The organic layer was washed with 3 x 200 mL water and was dried over 10 g anhydrous sodium sulfate. The solvent was removed by rotary evaporation and the residue was recrystallized in methylene chloride and hexane to give 150 mg product (54% yield). The product was confirmed by HPLC and the mass spectrometry (expected: 753; found: 753 M and 754 M+) **(Figure S1)**.

N-HPPH-lipid was synthesized by esterifying Boc-N-HPPH with 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-C16-PC, Avant #855675 P) at room temperature using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 4-dimethylaminopyridine in chloroform at a 1:1:2:2 lyso-C16-PC:Boc-N-HPPH:EDC:4-dimethylaminopyridine molar ratio for 24 hours. The product was confirmed by HPLC and mass spectrometry (expected: 1230; found: 616 (M+1)/2) (Figure S2). After the reaction, the chloroform was evaporated, 50% (v/v) trifluoroacetic and methylene chloride were added to remove Boc protection. The mixture was stirred for 3 hour at room temperature and solvent was removed by high vacuum. The crude product was purified by with diol silica gel and freeze dried in a solution of 80% t-butanol and 20% water. Purity was confirmed by HPLC (>95%) and identity was confirmed by mass spectrometry (expected: 1129; found: 1130 (M+1) and 566 (M+1)/2) (Figure S3). HPPH-lipid was synthesized as previously described. <sup>[2]</sup>

Mn-N-HPPH-lipid and Mn-HPPH-lipid were synthesized as previously described.<sup>[3]</sup> 20 excess folds of Mn acetate was incubated with the PoP for 30 hours in methanol in the dark. The solvent was removed and the metal-porphyrin lipid was extracted with chloroform:methanol:water 1:1.8:1, 3 times. For Mn-N-HPPH-lipid, chloroform:water 1:1 was used for extraction. The organic layer was collected and dried over anhydrous sodium sulfate. The solvent was removed by rotary evaporation and the product was freeze-dried as above.

Liposomes were formulated by the thin film method. After dissolving lipids in a borosilicate glass test tube, the organic solvent was evaporated by argon flow and the films were further dried under vacuum overnight. DD H<sub>2</sub>O or phosphate buffered saline (PBS) was added to hydrate the lipid films by sonication following freeze-thaw cycles. The liposome solution was extruded through a 100 nm polycarbonate

membrane with a handheld extruder. 50 mM sulforhodamine solution was used to hydrate the lipid film in the case of cargo-loaded liposomes. Free sulforhodamine was removed by a G-75 gel filtration column. The liposome fraction was collected and dialyzed overnight. The liposome formulations for optical absorption experiments were 100% PoP or 1:1 PoP:DMPC (molar ratio). For cryo-electron microscopy, 1:1 N-HPPH-lipid:DMPC (molar ratio) liposomes were used. For cargo release experiments, the liposome formulation was 2%-10% N-HPPH-lipid : 35% cholesterol : 63%-55% DMPC (adjusting the DMPC amount so that all lipids sum to 100 molar%). For *in vitro* experiments, unless otherwise noted, the liposome formulation was 1:1 PoP:DMPC and for *in vivo* experiments, the liposome formulation was 2% DMPC and for *in vivo* experiments, the liposome formulation was 2% DMPC and for *in vivo* experiments, the liposome formulation was 2% DMPC and for *in vivo* experiments, unless otherwise noted, the liposome formulation was 1:1 PoP:DMPC and for *in vivo* experiments, the liposome formulation was 2% DMPC and for *in vivo* experiments, the liposome formulation was 2% DMPC and for *in vivo* experiments, the liposome formulation was 2% DMPC and for *in vivo* experiments, the liposome formulation was 2% DMPC and for *in vivo* experiments, the liposome formulation was 2% DMPC and for *in vivo* experiments, the liposome formulation was 2% DMPC and for *in vivo* experiments, the liposome formulation was 45% PoP : 50% DMPC : 5% DSPE-PEG-2K (molar ratio). The lipid concentration for *in vivo* experiments was 20 mg/mL and for other experiment was 2 mg/mL.

## Molecular Dynamics (MD) simulations

Two membrane bilayer systems composed of HPPH-lipids and N-HPPH-lipids were simulated using classical MD. Each bilayer contains 128 lipid molecules (64 molecules per leaflet) of a single lipid type, i.e. either HPPH or N-HPPH. The topology of the HPPH-lipid molecule was inherited from our previously published simulation protocol<sup>[2]</sup> using the GROMOS 53a6 force-field<sup>[4]</sup>. This topology was then modified to accommodate the amino group NH3+ whose topology was reproduced from the similar amino group in the same force field. The HPPH and N-HPPH bilayer systems were fully solvated with 8986 and 10166 water molecules of Simple Point Charge (SPC) model, respectively.<sup>[5]</sup>

The hydrated bilayer systems underwent a careful equilibration process including a steepest decent procedure to minimize the system's potential energy, an NVT (constant particle number, volume and temperature) heating procedure with the Berendsen weak coupling thermostat<sup>[6]</sup> to gradually heat the whole system to the desired temperature of 310K over 500ps, an NVT annealing procedure to heat the membrane to 500K and then allow it to cool down to 310K while water positions were restrained over the course of 200ps. Then a non-restraint NPT (constant particle number, pressure and temperature) procedure of 500ps was applied to further equilibrate the whole system at the temperature of 310K and pressure of 1 bar using the v-rescale algorithm<sup>[7]</sup> and Parrinello-Rahman barostat<sup>[8]</sup>. After the above equilibration, a production run was carried out for 2.8 µs using the same NPT procedure as in the last step of the equilibration process.

All simulations were performed using the GROMACS software version 4<sup>[9]</sup> and the GROMOS 53a6 force-field<sup>[4]</sup>. Although CHARMM36 appears to be the best force field for lipids at this time, GROMOS 53a6 has been shown to perform well and, due to its united atom nature, is computationally much more efficient than the all-atom CHARMM36.<sup>[10]</sup> Periodic boundary conditions were applied in all directions. During the production phase, the Particle-Mesh Ewald method<sup>[11]</sup> was employed with a real space cutoff of 1.2 nm. The same cutoff was also used for the van der Waals interactions. The time-step was 2 fs. Membrane and water were coupled independently to the heat bath. The above equilibration and simulation protocols, and the applied methods have been used and tested by us and others.<sup>[12,13]</sup>

#### Cryo-electron microscopy

Holey carbon grids (c-flat CF-2/2-2C-T) were treated with chloroform for ~ 10 hours and then glow discharged at 5 mA for 15 seconds right before applying the sample to the grid. Approximately 3.4  $\mu$ L of 1:1 N-HPPH-lipid:DMPC liposomes at 2 mg/mL concentration in water were deposited directly on the electron microscopy grid. Grids were then blotted and plunged in liquid ethane at -180 °C using a Vitrobot (FEI). Temperature of the Vitrobot chamber was maintained at 25 °C and the relative humidity at 100% during the vitrification process. The grid was loaded into the FEI Tecnai F20 electron microscope operated at 200kV and a nominal magnification of 7,800X using a Gatan 626 single tilt cryo-holder.

Movies were captured with a Gatan K2 Summit direct detector device camera. This detector was used in counting movie mode with five electrons per pixel per second for 15 seconds exposures and 0.5 seconds per frame. This method produced movies consisting of 30 frames with an exposure rate of ~1  $e^{-}/Å^2$ . Movies were collected with a nominal defocus 3.5 µm and a nominal magnification of 7,800x, which produced images with a calibrated pixel size of 4.64 Å. Frames were aligned using the program alignframesleastsquares\_list.exe and averaged into one single micrograph with the shiftframes\_list.exe program from the Rubinstein group (<u>https://sites.google.com/site/rubinsteingroup/home</u>). These programs perform whole frame alignment of the movies using previously published motion correction algorithms.<sup>[14]</sup>

#### X-ray Diffraction

#### Preparation of Synthetic Membrane Samples

Highly oriented, multi-lamellar membranes were prepared on polished 1 x 1 cm<sup>2</sup> silicon wafers. The wafers were first pre-treated by sonication in dichloromethane (DCM) at 310 K for 25 minutes to remove all organic contamination and to create a hydrophobic substrate. After removal from the DCM post-sonication, each wafer was thoroughly rinsed three times by alternating with ~50 mL of ultra-pure water (18 M $\Omega$  cm) and methanol. Lipid films of HPPH-lipid, N-HPPH-lipid, 1:1 HPPH-lipid:DMPC, and 1:1 N-HPPH-lipid: DMPC (molar ratios) were dissolved in 1:1 mixture of trifluoroethanol (TFE):chloroform at a concentration of 15 mg/mL.

A tilting incubator was heated to 313 K and the lipid solutions placed inside to equilibrate. 85  $\mu$ L of lipid solution was deposited on each wafer and the solvent was then allowed to slowly evaporate for ~10 minutes while being gently rocked, such that the lipid solution spread evenly on the wafers. After drying, the membrane samples were placed in vacuum at 313 K for 12 hours to remove all traces of solvent. About 3000 highly oriented stacked membranes with a total thickness of ~10  $\mu$ m are typically produced using this protocol.<sup>[15–18]</sup>

### X-ray scattering experiments

Out-of-plane X-ray scattering data was obtained using the Biological Large Angle Diffraction Experiment (BLADE) in the Laboratory for Membrane and Protein Dynamics at McMaster University. BLADE uses

a 9kW (45 kV, 200 mA) CuK-α Rigaku Smartlab rotating anode at a wavelength of 0.15418 nm. Both source and detector are mounted on moveable arms, such that the membranes stay horizontal during measurements. Focussing, multi-layer optics provide a high intensity parallel beam with monochromatic X-ray intensities up to 10<sup>10</sup> counts/(s mm<sup>2</sup>). This beam geometry provides optimal illumination of the membrane samples to maximize the scattered signal.

Samples were placed in a temperature and humidity controlled chamber during scanning. The chamber contained a trough with a saturated solution of either K<sub>2</sub>SO<sub>4</sub> or NaCl to produce atmospheres with 97% and 75% relative humidity (RH), respectively. Out-of-plane diffraction measurements were then taken of each sample. Initially, quick scans (10 minutes in duration) were taken to observe the water uptake of the membranes. After equilibration, longer scans were acquired to obtain higher resolution data for structural analysis.

### Out-of-plane structure and electron densities

The out-of-plane structure of the membranes was determined using the out-of-plane X-ray diffraction in Fig. S8c. The membrane electron density,  $\rho(z)$ , is approximated by a 1-dimensional Fourier analysis<sup>[19,20]</sup>:

$$\rho(z) = \frac{2}{d_z} \sum_{n=1}^{N} F(q_n) \cos(\frac{2\pi nz}{d_z})$$
$$= \frac{2}{d_z} \sum_{n=1}^{N} \sqrt{I_n q_n} \nu_n \cos(\frac{2\pi nz}{d_z}), \qquad (1)$$

where *N* is the highest order of the Bragg peaks observed in the experiment. The integrated peak intensities,  $I_n$ , are multiplied by  $q_n$  to generate the form factors,  $F(q_n)$ . The bilayer form factor, which is in general a complex quantity, is real-valued when the structure is centro-symmetric. The phase problem of crystallography, therefore, simplifies to the sign problem  $F(q_z) = \pm |F(q_z)|$  and the phases,  $v_n$ , can only take the values  $\pm 1$ . To determine the phases for this study, a fitting procedure was used, as described previously.<sup>[16,20,21]</sup>

The calculated electron densities,  $\rho(z)$ , which are initially on an arbitrary scale, were then scaled until the total number of electrons, e<sup>-</sup>, across a membrane leaflet,  $e^- = A_L \int_0^{d_Z/2} \rho(z) dz$  agreed with the total number of electrons calculated based on the sample composition. The spacing between two neighbouring membranes in the stack, d<sub>z</sub>, was determined from the distance between the Bragg reflections (d<sub>z</sub> =  $2\pi/\Delta q_z$ ) along the out-of-plane axis, q<sub>z</sub>.

#### Electron spin resonance (ESR) spectroscopy

Spin-labeled phosphatidylcholines were incorporated in mixed bilayer membranes of DMPC and PoP at a relative concentration of 0.5 mol% by drying down the lipid solutions in chloroform/methanol and then suspending the dry lipid in water or appropriate salt solution above the chain melt temperature for at least 10 min. The DMPC:HPPH-lipid or DMPC:N-HPPH-lipid ratio in samples studied varied from 1:1 to  $\sim$  12:1. Aliquots of the dispersions containing 1 mg of the lipid were transferred into 50 µL, 0.7 mm inner diameter glass capillaries and spun down for 10 min at 10000g. The centrifuge was supplied with a capillary (microhematocrit) rotor.

The ESR spectra from the labeled lipids were analyzed using the NLLS fitting program based on the stochastic Liouville equation<sup>[22,23]</sup> using the MOMD or Microscopic Order Macroscopic Disorder model as in previous studies<sup>[24,25]</sup>. The magnetic A-tensor and g-tensor components for PC spin labels were used as in<sup>[25]</sup>.

#### Liposome stability and cargo release

The porphyrin liposome stability was based on the porphyrin molecule fluorescence quenching. The fluorescence quenching equaled to  $1-(F_{final} - F_{initial}) / F_{Tx-100}$ . Cargo release was based on sulforhodamine B fluorescence. The cargo fluorescence was quenched when loaded in the liposome and recovered after adding Triton X-100 (TX-100) detergent. Fluorescence was measured in a Tecan microplate reader. For N-HPPH-lipid cargo release, the fluorescence of sulforhodamine B was measured before and after 24h dialysis in PBS. Before measurement, TX-100 was added to unquench the sulforhodamine B fluorescence and calculate release percentages. For the 2% N-HPPH-liposome loading stability experiment, incubation conditions were in PBS or 50% fetal bovine serum. The fluorescence of sulforhodamine B was measured at 24h and 48h.

For liposome stability in various buffers, liposomes formed with a molar ratio of [50:5:45] [DMPC:DSPE-PEG2K:PoP] were incubated in different buffers and the fluorescence of samples was measured at different time points. Triton X-100 was added finally to determine the fluorescence of fully disrupted structures

#### **Biological studies**

Animal studies were carried out in accordance with IACUC protocols of Roswell Park Cancer Institute and University at Buffalo.

#### MR imaging

Magnetic resonance images were acquired on a 4.7 Tesla preclinical scanner using the ParaVision 3.0.2 platform (Bruker Biospin). In vitro T1 relaxation rates were measured at 37 °C using an inversion-recovery, balanced steady-state free precession scan as described previously.<sup>[26]</sup> A custom-built 35 mm (ID) radiofrequency coil (m2m Imaging) was used to encompass the thorax and abdomen of the mice. Mice were anesthetized with isoflurane and body temperature and respiration were monitored with an MR-compatible monitoring system (SA Instruments). Two NMR tubes containing 1 mM and 2 mM CuSO<sub>4</sub> in 1% agarose were included for inter-scan signal normalization. Following localizer scans, a T1-weighted, spoiled-gradient echo scan (TE/TR/flip angle = 3/15/40, field of view = 48x32x32 mm, matrix = 192x96x96, NEX=2) was acquired prior to administration of Mn-N-HPPH-liposome to provide baseline intensity values. Mn-H-HPPH-liposome were injected intravenously at a concentration of 0.1 mmol/kg and mice were re-imaged immediately, 6 hrs and 24hrs after administration. Imaging data were reconstructed to isotropic voxel size of 187 micron and processed on commercially available software (Analyze 7.0, AnalyzeDirect). Regions of interests were drawn for the inferior vena cava and liver and intensity values sampled for each time point.

#### Pharmacokinetics

Female BALB/c mice (18–20 g) were intravenously injected via tail vein with 200  $\mu$ L of 20 mg/ml N-HPPH-lipid liposomes (DMPC:N-HPPH-lipid:DSPE-PEG2000 50:45:5), n = 5. Small blood volumes

were sampled at 0.5, 2, 4, 9, 24, and 48 h post-injection. Serum was collected and diluted 100 times in PBS buffer (1% Triton X-100). The final solution samples was measured and analyzed by fluorescence. The N-HPPH-lipid concentrationa was determined by a standard curve. Noncomparamental pharmacokinetics parameters were analyzed by PKsolver software.

## Toxicity

In vitro toxicity was assessed with Caco-2 cells that were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 1% penicillin/ streptomycin at 5% CO2. Caco-2 cells were seeded at  $2x10^4$  cells per well in a 96-well plate and allowed to adhere for 24 hours. Liposomes composed of various lipids were added to wells at the indicated concentration. Cells maintained in DMEM with FBS were incubated with liposomes for 48h. After 48 hours, media was removed and cells were washed with PBS once. 100 µl of PBS containing XTT at 50 µg/mL and PMS at 30 µg/mL was added to each well and incubated for 2h at 37 °C. Absorbance of the XTT treated wells was measured at 450 nm. Reference was based on the absorbance measurement at 630 nm. Cell viability was calculated as the ratio of treated wells to untreated wells. Average of triplicate data was used to calculate cell viability. Error bars indicated standard deviation with respect to the average cell viability. For in vivo acute toxicity, female BALB/C was (16-19 g) were intravenously injected via tail vein with 200 ul 20 mg/ml N-HPPH-liposome (DMPC:N-HPPH-lipid:DSPE-PEG2000 50:45:5) or PBS buffer, n = 5. The body weight of mice was monitored and measured at day 1, 3, 6, 8 and 10 post-injection.

## **Supplementary References**

- [1] A. J. Pallenberg, M. P. Dobhal, R. K. Pandey, Org. Process Res. Dev. 2004, 8, 287.
- [2] K. A. Carter, S. Shao, M. I. Hoopes, D. Luo, B. Ahsan, V. M. Grigoryants, W. Song, H. Huang, G. Zhang, R. K. Pandey, J. Geng, B. A. Pfeifer, C. P. Scholes, J. Ortega, M. Karttunen, J. F. Lovell, *Nat. Commun.* 2014, 5, DOI 10.1038/ncomms4546.
- [3] S. Shao, J. Geng, H. Ah Yi, S. Gogia, S. Neelamegham, A. Jacobs, J. F. Lovell, *Nat Chem* **2015**, *7*, 438.
- [4] C. Oostenbrink, T. A. Soares, N. F. A. van der Vegt, W. F. van Gunsteren, *Eur. Biophys. J. EBJ* 2005, *34*, 273.
- [5] H. J. C. Berendsen, J. P. M. Postma, W. F. van Gunsteren, Hermans.J, *Intermolecular Forces* **1981**, 331.
- [6] H. J. C. Berendsen, J. P. M. Postma, W. F. van Gunsteren, A. DiNola, J. R. Haak, J. Chem. Phys. 1984, 81, 3684.
- [7] G. Bussi, D. Donadio, M. Parrinello, J. Chem. Phys. 2007, 126, 14101.
- [8] M. Parrinello, A. Rahman, J. Appl. Phys. 1981, 52, 7182.
- [9] B. Hess, C. Kutzner, D. van der Spoel, E. Lindahl, J. Chem. Theory Comput. 2008, 4, 435.
- [10] J. B. Klauda, R. M. Venable, J. A. Freites, J. W. O'Connor, D. J. Tobias, C. Mondragon-Ramirez, I. Vorobyov, A. D. MacKerell, R. W. Pastor, J. Phys. Chem. B 2010, 114, 7830.
- [11] T. Darden, D. York, L. Pedersen, J. Chem. Phys. 1993, 98, 10089.
- [12] J. Wong-Ekkabut, M. Karttunen, J. Chem. Theory Comput. 2012, 8, 2905.
- [13] W. F. D. Bennett, N. Sapay, D. P. Tieleman, Biophys. J. 2014, 106, 210.
- [14] X. Li, P. Mooney, S. Zheng, C. R. Booth, M. B. Braunfeld, S. Gubbens, D. A. Agard, Y. Cheng, *Nat. Methods* 2013, 10, 584.

- [15] R. J. Alsop, A. Khondker, J. S. Hub, M. C. Rheinstädter, Sci. Rep. 2016, 6, DOI 10.1038/srep22425.
- [16] R. J. Alsop, R. M. Schober, M. C. Rheinstädter, Soft Matter 2016, 12, 6737.
- [17] H. Dies, L. Toppozini, M. C. Rheinstädter, PLOS ONE 2014, 9, e99124.
- [18] J. Tang, R. J. Alsop, M. Backholm, H. Dies, A.-C. Shi, M. C. Rheinstädter, *Soft Matter* 2016, 12, 3165.
- [19] T. Adachi, Chem. Phys. Lipids 2000, 107, 93.
- [20] M. A. Barrett, S. Zheng, G. Roshankar, R. J. Alsop, R. K. R. Belanger, C. Huynh, N. Kučerka, M. C. Rheinstädter, *PLOS ONE* 2012, 7, e34357.
- [21] R. J. Alsop, C. L. Armstrong, A. Maqbool, L. Toppozini, H. Dies, M. C. Rheinstädter, *Soft Matter* **2015**, *11*, 4756.
- [22] D. J. Schneider, J. H. Freed, in *Spin Labeling* (Eds: L.J. Berliner, J. Reuben), Springer US, **1989**, pp. 1–76.
- [23] D. E. Budil, S. Lee, S. Saxena, J. H. Freed, J. Magn. Reson. A 1996, 120, 155.
- [24] M. Ge, J. H. Freed, Biophys. J. 2003, 85, 4023.
- [25] M. Ge, J. H. Freed, Biophys. J. 2009, 96, 4925.
- [26] S. J. Dorazio, P. B. Tsitovich, K. E. Siters, J. A. Spernyak, J. R. Morrow, J. Am. Chem. Soc. 2011, 133, 14154.

## **Supplementary Figures**



Figure S1: Synthesis of pyropheophorbide-*a* to Boc-N-HPPH. HPLC spectrum and MS spectrum are shown.



Figure S2: Synthesis of Boc-N-HPPH to Boc-N-HPPH-lipid. HPLC spectrum and MS spectrum are shown.





Figure S3: Deprotection of Boc group. The HPLC and MS spectrum of the final product, N-HPPH-lipid, are shown.



**Figure S4:** Absorbance of N-HPPH lipid, Mn-N-HPPH-lipid, N-HPPH-lipid liposomes and Mn-N-HPPH-lipid liposomes. The liposome was formulated with PoP and DMPC at a 1:1 molar ratio. The lipids were measured in chloroform and the liposomes were measured in water. Spectra were normalized by the Soret band peak.



Figure S5: Total electron density based on MD simulations of HPPH-lipid and N-HPPH-lipid bilayers.



Figure S6: Amount of soluble PoP following water addition and brief vortexing of lipid films composed of indicated lipids.



Figure S7: Dynamic light scattering of N-HPPH-lipid liposomes.



**Figure S8:** Out-of plane X-ray diffraction measurements of oriented membrane samples. Molar ratios of PoP and DMPC compositions are indicated, as well as the relative humidities (RH).

Defau NHHPI	<b>ult file</b> PHLIPOSOME5 1	2 15								3: Diode A	Arraγ
∎∩	1.0e-1	- 1.81		5.29 6.61	8.06					Range: 1.26	660 6e-1
NHHP	PHLIPOSOME5_1	2.00 2_15(2)	4.00	6.00	8.00 8.08	10.00	12.00	14.00	16.00	18.00 20.0 2: Diode A	0 Array 660
AU	0.0	1.83		5.30 6.61	$\underline{\Lambda}$					Range: 1.22	?6e-1
NHHP	PHLIPOSOME5_1	2.00 2_15(3)	4.00	6.00	8.00	10.00	12.00	14.00	16.00	18.00 20.0 2: Diode A	0 Array
AU	5.0e-2	1.82_2.12		5.29 6.61	8.07					Range: 1.15	660 i3e-1
NHHP	U.U <sup>.</sup>	2.00 2 15(4)	4.00	6.00	8.00	10.00	12.00	14.00	16.00	18.00 20.0 4: Diode A	0 Arrav
٩U	5.0e-2 0.52	2.12		5.29 6.59	8.05					Range: 1.11	660 5e-1
NHHP	U.U-++++++ PHLIPOSOME5_1	2.00 2_15(6)	4.00	6.00	8.00	10.00	12.00	14.00	16.00	18.00 20.0 5: Diode A	0 Array
AU	1.0e-1 0.20			5.30 _ 6.60	8.08					Range: 1.23	660 }6e-1
NHHP	PHLIPOSOME5_1	2.00 2_15(8)	4.00	6.00	8.00	10.00	12.00	14.00	16.00	18.00 20.0 4: Diode A	0 Array
AU	0.14	2.20		5.30 6.60	8.08					Range: 1.15	660 54e-1
NHHP	U.U <del>.S</del> PHLIPOSOME5 1	2.00 2 15(10)	4.00	6.00	8.00	10.00	12.00	14.00	16.00	18.00 20.0 3: Diode A	0 Array
ΑU	-			5 29 6 59	8.07					Range: 1.97	660 77e-1
`	0.0 <b>4,,,,,</b>	2.00	4.00		<del></del>	10.00	12.00	14.00	16.00		íime O

2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20 **Figure S9**: HPLC traces of N-HPPH-liposomes (1:1 DMPC:PoP, molar ratio) stored in water at 4 °C.



**24h 48h Figure S10:** Loading stability of 2% N-HPPH-liposome in PBS and 50% FBS. Sulforhodamine B was used as the cargo.



**Figure S11:** Unquenching of the indicated PoP liposomes in various buffers over time. The liposome formulation was PoP:DMPC 1:1 (molar ratio).



Figure S12: Stability of Mn chelation during incubation in indicated buffers over time. If Mn was to exit the PoP, fluorescence restoration would be observed. The liposome formulation was PoP:DMPC, 1:1



**Figure S13:** Inverse relaxition plot for r2 of N-HPPH-lipid and HPPH-lipid liposomes. Liposomes were formed with 1:1 PoP:DMPC (molar ratio).



N-HPPH-lipid Mn-N-HPPH-lipid HPPH-lipid Mn-HPPH-lipid Figure S14: In vitro cytotoxicity of various PoP and Mn-PoP liposomes (PoP:DMPC molar ratio of 1:1). Caco-2 cells were incubated with liposomes at indicated concentrations for 48 hours and the XTT viability assay was then performed.



100

t <sub>1/2</sub> (h)	8.21±1.79
C <sub>max</sub> (µmol/L)	25.95±7.20
AUC <sub>0-Inf</sub> (µmol/L*h)	119.03±42.79
MRT <sub>0-Inf</sub> (h)	8.38±1.55
CI (ml/h/mmol)	0.76±0.31
V <sub>ss</sub> (mL/mmol)	6.42±3.30

**Figure S15:** Blood circulation parameters of N-HPPH-lipid in liposomes [PoP:DMPC:DSPE-PEG-2K] [45:50:5], following intravenous injection of 120 mg/kg N-HPPH-lipid. Data show mean +/ std. dev. for n=5 mice per group



**Figure S16:** Mouse body weights following intravenous injection of N-HPPH-lipid [PoP:DMPC:DSPE-PEG 2k] [45:50:5], 120 mg/kg PoP. Data show mean +/- std. dev. for n=5 mice per group.