Supplementary Information for

The Self-Assembly and Secondary Structure of Peptide Amphiphiles Determine the Membrane Permeation Activity

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1. Materials.

N-9-Fluorenylmethoxycarbonyl (Fmoc) protected L-amino acids (Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Trp(Boc)-OH), 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazol (HOBt), *N*,*N*-diisopropylethylamine (DIEA), 20% (v/v) piperidine in *N*,*N*-dimethylformamide, trifluoroacetic acid (TFA) and triisopropyl silane (TIPS) were purchased from Watanabe Chemical Industry (Hiroshima, Japan). Rink amide AM resin was purchased from EMD Millipore (Billerica, MA, USA). Palmitic acid, dichloromethane (DCM), methanol and acetonitrile were purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). *N*,*N*-dimethylformamide (DMF) and diethylether were purchased from Wako Pure Chemical Industries (Osaka, Japan). α-cyano-4-hydroxycinnamic acid (α-CHCA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphatidyl choline was purchased from NOF Co. (Tokyo, Japan). Calcein and cell counting kit-8 were purchased from Dojindo Laboratories (Kumamoto, Japan). F-12 Nutrient Mixture (Ham's F-12), fetal bovine serum (FBS), penicillin, streptomycin, Opti-MEM and Dulbecco's phosphate-buffered saline (D-PBS), were purchased from Invitrogen (GIBCO, Carlsbad, CA, USA).

2. General methods

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) was performed on an Autoflex-III (Bruker, Billerica, MA, USA) using α-CHCA as the matrix. Circular dichroism (CD) spectra were recorded on a J-725G spectropolarimeter (JASCO, Tokyo, Japan). Fluorescence spectra were measured on either a LS55C or a LS55KG spectrometer (Perkin Elmer, Waltham, MA, USA). Transmission electron microscopy (TEM) was performed on a TEM-2010 (JEOL, Tokyo, Japan).

3. Synthesis of PA1 and PA2

PA1 and **PA2** were synthesized at a 0.1-mmol scale on Rink Amide AM resin using standard Fmoc solid-phase peptide synthesis (SPPS).[1] Briefly, to the pre-equilibrated resin with DMF in a reaction tube was added a solution of 20% piperidine in DMF. After shaking for 20 min, the solution was drained off and the resin was washed with DMF (3 times) and DCM (3 times). The presence of free amino groups was indicated by a blue color in the Kaiser test. DIEA (6 equiv) was added to a DMF solution of Fmoc-amino acids (4 equiv relative to resin loading), HBTU (3.9 equiv) and HOBt (4 equiv), and the mixture was stirred for 1 min for activation. The solution mixture was added to the resin and shaken for 0.5–1 h, drained, and the peptidyl resin was washed with DMF (3 times) and DCM (3 times). The Kaiser test showed a yellow color indicative of the absence of free amino groups. After all amino acids were conjugated to the resin, palmitic acid was reacted in the same manner as the Fmoc-amino acids. The cleavage of PAs from the resin and deprotection of the protective groups on side chains were performed with a cleavage cocktail of TFA/TIPS/H_2O (95/2.5/2.5). After shaking for 2 h, crude PAs were precipitated by adding cold ether. The precipitate was collected by centrifugation (3,000 g, 5 min), washed with cold ether (5 times) and dried under vacuum overnight. The crude PAs were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) on a JASCO LC-2000 Plus HPLC system equipped with a JASCO PU-2086 preparative pump and a UV-2070 UV-Vis detector using an Inertsil ODS-3 column (10 × 250 mm, GL Science). The purified PAs were lyophilized and characterized by MALDI TOF MS: *m*/z: **PA1**: calcd for $C_{50}H_{89}N_9O_{13}$: 1023.66; found: 1022.5 $[M–H]^+$; 1044.5 $[M–H+Na]^+$; **PA2**: calcd for $C_{83}H_{134}N_{16}O_{23}$: 1722.98; found: 1722.3 $[M–H]^+$: see Figure S1.

4. MALDI TOF MS spectra of PA1 and PA2

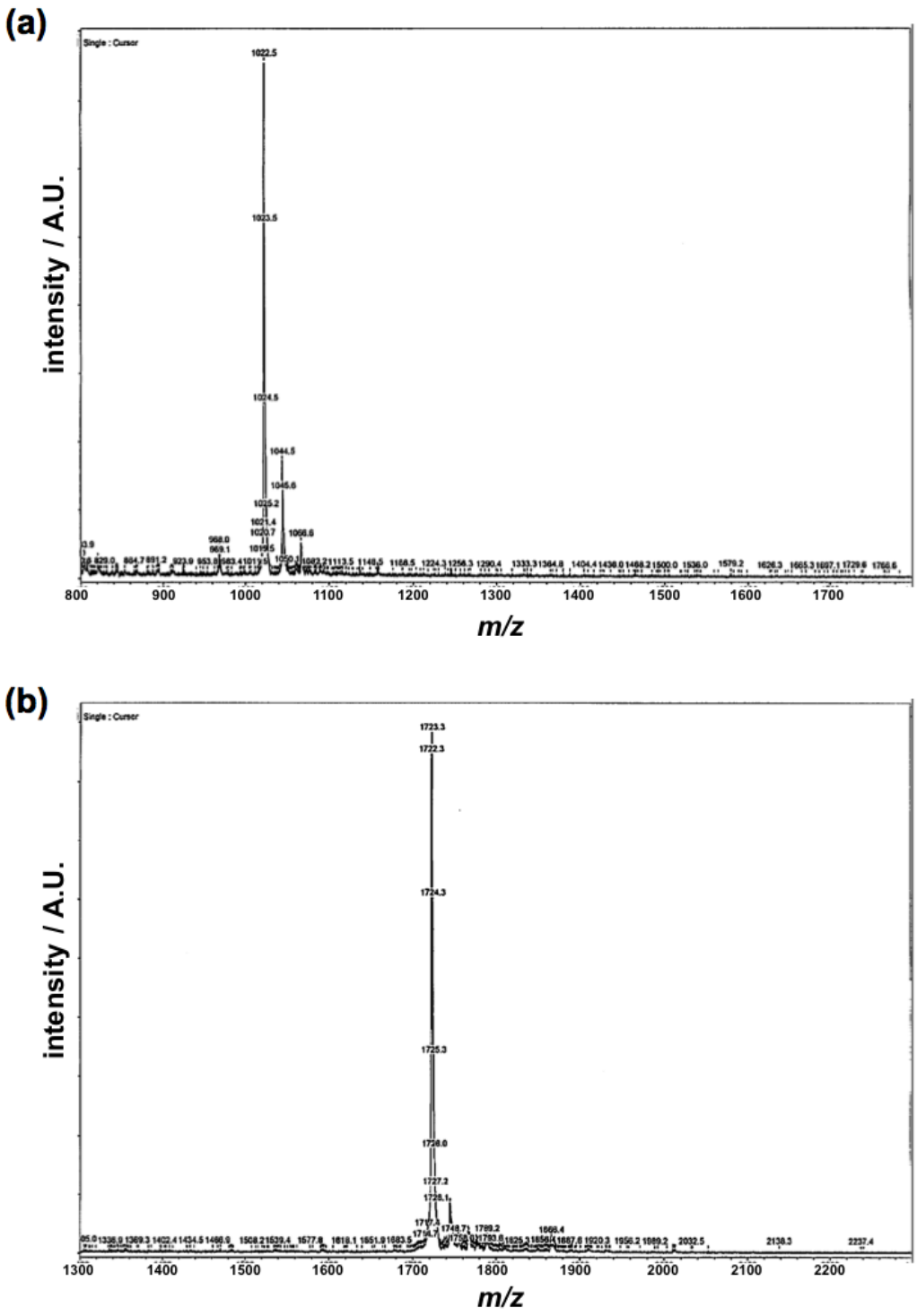

Figure S1. MALDI TOF MS spectra of (a) **PA1** and (b) **PA2**.

5. Critical micelle concentration (CMC) measurements of PA1 and PA2

The CMC of the PAs was measured using the pyrene solubilizing method.[2] PA aqueous solutions with different concentrations (0–200 μM) in 10 mM phosphate buffer (pH 5.5 or 7.5) in the presence of pyrene (1 μM) were incubated at 37 °C for 2 h. Emission intensities at 373 nm (I_1) and 385 nm (I_3) were recorded at varying PA concentrations (λ_{em} = 334 nm) on a Perkin Elmer LS55KG spectrometer using a 96-well black plate.

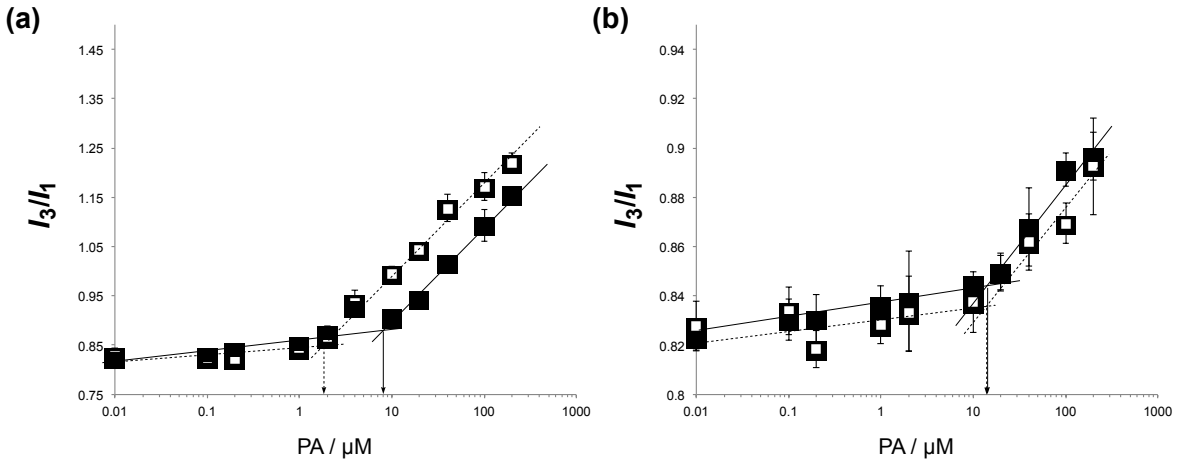

Figure S2. CMC measurements of (a) **PA1** and (b) **PA2** at pH 5.5 (filled) and pH 7.5 (open). The ratio of fluorescence intensities at 385 nm (I_3) and 373 nm (I_1) against PA concentrations was plotted (λ_{ex} = 334 nm). Data are mean ± SD from representative runs.

6. Transmission electron microscopy (TEM) of PA1 and PA2

Aqueous solutions of PAs (100 μM) in 10 mM phosphate buffer (pH 5.5 or 7.5) were prepared and incubated for 1 h. Two microliters of the solution was deposited onto a carbon-coated copper grid (Okenshoji Co., Ltd.), incubated for 1 min and the excess liquid was absorbed by a filter paper. The sample was negatively stained with a 2% uranyl acetate solution and allowed to dry under vacuum prior to imaging. All TEM images were obtained using a JEOL TEM-2010 at an accelerating voltage of 120 kV. Nanofibers were observed for **PA1** at pH 5.5 and 7.5 (See Figure S3), but no distinct nanostructures were found for **PA2**.

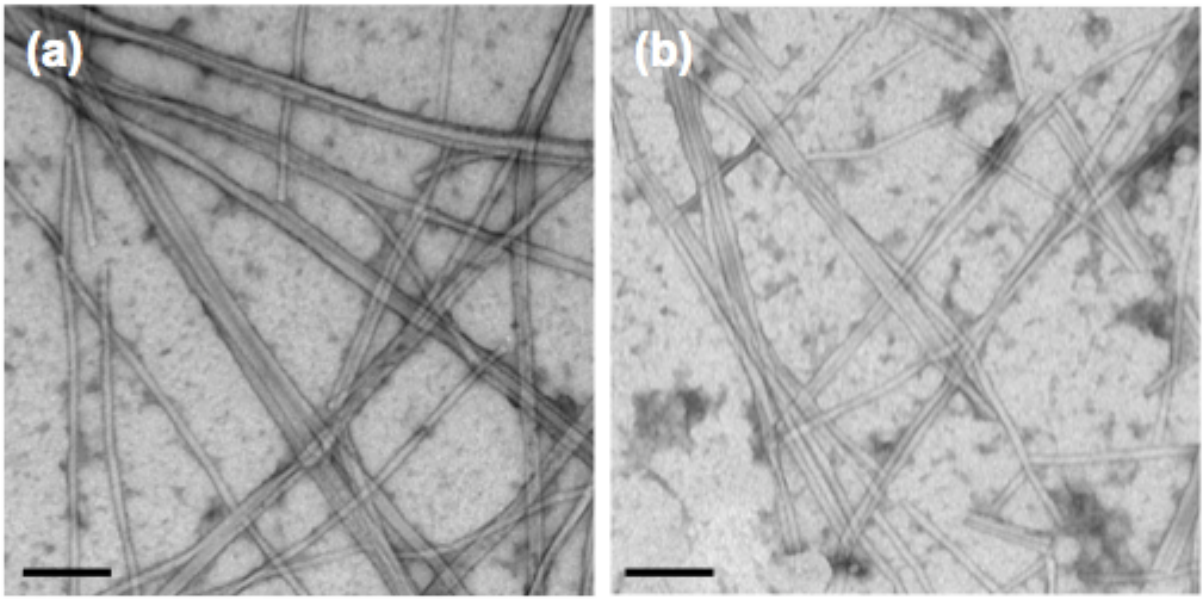

Figure S3. TEM images of **PA1** at (a) pH 5.5 and (b) pH 7.5 ([**PA1**] = 100 μM, scale bar: 100 nm).

7. Liposomal leakage by PAs

Liposomes composed of egg PC (EPC) were prepared by the film hydration method.[3] A chloroform solution of EPC was added to a test tube and the solvent was evaporated using an N_2 flow to yield a thin lipid film. The film was further dried under vacuum overnight. The dried film was hydrated with 10 mM phosphate buffer containing 100 mM calcein (above self-quenching concentration) at 37 °C for 2 h with mixing every 10 min. The resultant liposome was then treated with freeze-thaw cycles (10 times). Non-encapsulated calcein was removed by dialysis using 12–14 kDa molecular weight cutoff membrane tubes (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) against 10 mM phosphate buffer (4 times). The concentration of EPC in the resultant liposomal suspension was measured by a colorimetric method described previously.[4]

The above-mentioned liposomal suspension was diluted to 100 μM EPC with 10 mM phosphate buffer (pH 5.5 or 7.5). Three microliters of PA solutions varying in concentration were added to 300-μL liposomal suspensions and the solutions were incubated at 37 °C for 2 h. The fluorescence intensity at 520 nm (λ_{em} = 395 nm) was measured with a Perkin Elmer LS55C. Calcein release was calculated using the following equation:

$$\% \text{ leakage} = (I - I_0) / (I_{max} - I_0)$$

where I_0 and I_{max} are the fluorescence intensities of the control samples without the addition of the PAs and with the addition of Triton X-100, respectively. The calcein release was plotted against various PA/EPC ratios (see Figure 2).

8. Dynamic light scattering (DLS) of liposomes treated with PAs

Liposomal suspensions were treated with PAs at a PA/EPC ratio of 1/100 as described before and DLS was performed using a Zetasizer Nano-Zs (Malvern, Worcestershire, UK). The relative light scattering intensities are shown in Figure S4 and the hydrodynamic diameters combined with the calcein release activities of PAs are summarized in Table S1.

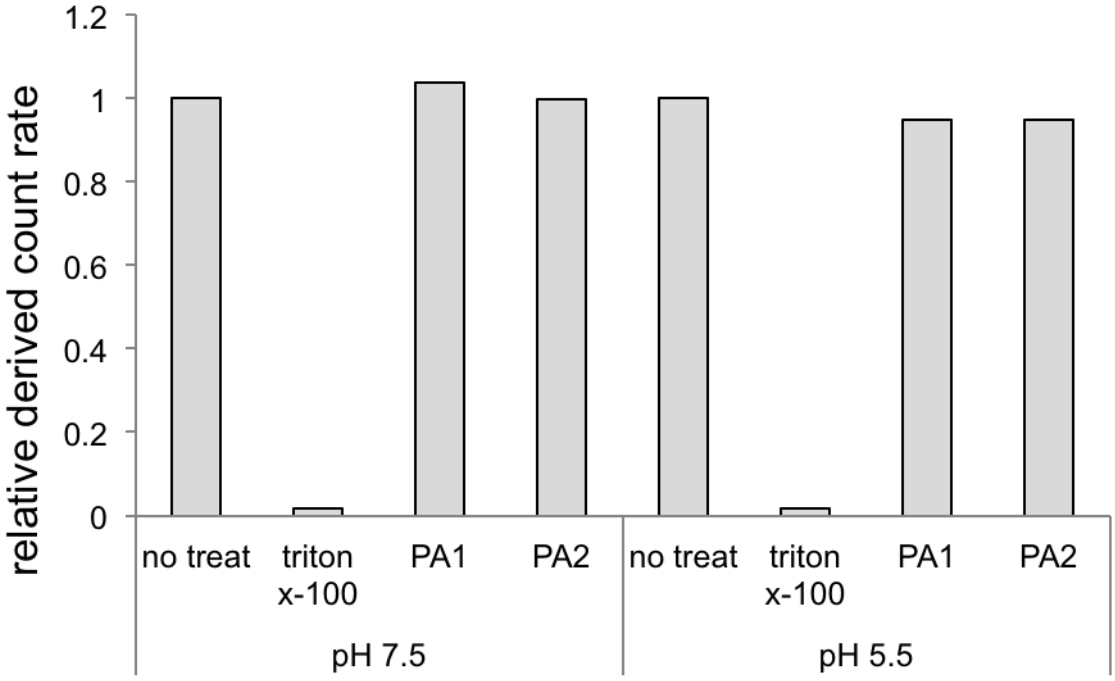

Figure S4. DLS measurement of liposome solutions treated with PAs (PA/EPC = 1/100, [PA] = 1 μM, [EPC] = 100 μM).

Table S1. Calcein leakage and hydrodynamic diameter of liposomes determined by fluorescence measurement and DLS, respectively ([PC] = 100 μM, [PA] = 1 μM, [triton X-100] = 0.1 wt%).

	pH 5.5		pH 7.5	
	calcein leakage / %	size, d / nm	calcein leakage / %	size, d / nm
no treatment	0	129.4	0	128.9
triton x-100	100	8.0*	100	8.7*
PA1	21	130.2	3	128.9
PA2	>99	129.0	55	129.2

*Micelles formed by triton x-100

9. Cytotoxicity of PAs

A Chinese Hamster Ovary (CHO) cell line was purchased from the Riken Bioresource Center (Ibaraki, Japan). CHO cells were cultivated in Ham's F12 supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were cultured with 5% CO_2 at 37 °C. For the cytotoxicity assay, the cells were seeded on a 96-well multi-well plate (4,000 cells/well) and incubated overnight in Ham's F12 containing 10% FBS. The medium was removed and the cells were washed with D-PBS. Subsequently, PA solutions of varying PA concentrations in Opti-MEM were added and incubated for 24 h. Cell viability was determined by the WST assay using a cell counting kit (Dojindo). Relative cellular viability was calculated using a control sample that was not treated with the PAs (see Figure 3).

References

1. Chan, W.C., White, P.D. Eds., *Fmoc Solid Phase Peptide Synthesis: A Practical Approach,* Oxford University Press, New York, 2000.
2. Ananthapadmanabhan, K. P., Goddard, E. D., Turro, N. J., Kuo, P. L. *Langmuir* **1985**, *1*, 352–355.
3. Dua, J. S., Rana, A. C., Bhandari, A. K. *Int. J. Pharm. Studies Res.* **2012**, *3*, 14–20.
4. Stewart, J. C. M. *Anal. Biochem.* **1980**, *104*, 10–14.