Supplementary Information

Stabilization of bicelles using metal-binding peptide for extended blood circulation

Yuichiro Takagi,[a] Noriyuki Uchida,[*a] Yasutaka Anraku,[bc] and Takahiro Muraoka[*ade]

[a.] *Department of Applied Chemistry, Graduate School of Engineering, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588, Japan.*

[b.] *Department of Bioengineering, Graduate School of Engineering, The University of Tokyo7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan.*

[c.] *Innovation Center of Nanomedicine Kawasaki Institute of Industrial Promotion3-25-14, Tonomachi, Kawasaki-ku, Kawasaki 210-0821, Japan.*

[d.] *Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, 3-8-1 Harumi-cho, Fuchu-shi, Tokyo 183-8538, Japan.*

[e.] *Kanagawa Institute of Industrial Science and Technology, 705-1 Shimoimaizumi, Ebina, Kanagawa 243-0435, Japan.*

Table of Contents

1. Materials

Cholic acid and *N*,*N*-diisopropylethylamine (DIEA) were purchased from Nacalai Tesque (Kyoto, Japan). Acetonitrile, *N*,*N'*-dimethylformamide (DMF), Et_2O, *N*-methyl-2-pyrrolidone (NMP), piperidine, and trifluoroacetic acid (TFA) were purchased from Kishida Chemical (Tokyo, Japan). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine (DPPE), 2,4,6-trinitrobenzene sulfonic acid (TNBS) Test Kit and triisopropylsilane (TIS) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Fmoc-His(Trt)-OH, Fmoc-Gly-OH, Fmoc-NH-SAL Resin, Fmoc-Pro-OH, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 1,2,3-benzotriazol-1-ol monohydrate ($HOBt•H_2O$) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). Deionized water (filtered through a 0.22 μm membrane filter, >18.2 MΩ cm) was purified in Purelab DV35 of ELGA (Buckinghamshire, UK) and a Milli-Q system of Merck Millipore (Burlington, MA, USA). Phosphate buffered saline (D-PBS) and Cell Counting Kit-8 were purchased from Wako (Tokyo, Japan).

2. Instrumentation

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS) was performed on autoflex speed spectrometer (Bruker, Bremen, Germany) in a reflector positive mode using 2,5-dihydroxybenzoic acid as a matrix. Circular dichroism spectra were recorded on J-1100 CD spectrometer (JASCO, Tokyo, Japan) with PTC-514 peltier temperature controller. Transmission electron microscopic (TEM) observations were performed with JEM-1400 (JEOL, Tokyo, Japan) equipped with Gtan UltraScan 4k×4k CCD camera (Gatan, CA, USA) using carbon reinforced microgrid (Cu 200 mesh) and $Gd(CH_3CO_2)_3$ as a stainer under 120 kV accelerating voltage, and a photographing method of JEOL minimum dose system was utilized (MDS, search/focus/record modes). ^{31}P nuclear magnetic resonance (NMR) spectra were recorded on ECX-400 spectrometer (JEOL, Tokyo, Japan) or an Ascend-600 spectrometer (Bruker, Bremen, Germany). Small angle X-ray scattering (SAXS) measurements were performed on NANOPIX 3.5m system (Rigaku, Tokyo, Japan). Intravital confocal laser scanning microscopy (IV-CLSM) was performed with a Nikon A1R. Peptide syntheses were conducted with a shaking apparatus PetiSyzer PSP-5100 of HiPep Laboratories (Kyoto, Japan) using polypropylene LibraTubes with a filter. Lyophilization was conducted with FDU-1200 lyophilizer (EYELA, Tokyo, Japan). Centrifugation was conducted with a micro refrigerated centrifuge 3700 (Kubota, Tokyo, Japan).

3. Syntheses

MBP and Chol-MBP were synthesized by Fmoc solid-phase peptide synthesis. Typically, a condensation-reagents cocktail of HBTU (2.28 g, 6.01 mmol) and HOBt-H_2O (0.94 g, 6.12 mmol) in DMF (12 mL), a mixture of DIEA (2.24 mL) and NMP (12 mL), TIS (125 μL), a cleavage cocktail of TFA (4.75 mL) and water (125 μL) was prepared just before synthesis. Fmoc-NH-SAL Resin (0.08 mmol) in a polypropylene tube was soaked in DMF (2 mL) over 3 h at 25 °C. After removal of DMF, piperidine in DMF (20%, 2 mL) was added and mixed with a vortex device for 1 min. After the reaction solution was removed, piperidine in DMF (20%, 2 mL) was added and the reaction tube was shaken for 10 min at 25 °C. After removal of the reaction solution, the resin was washed with DMF (2 mL, 5 times), CH_2Cl_2 (2 mL, 3 times) and DMF (2 mL, 3 times). To the resin was added Fmoc-protected amino acid (0.24 mmol) dissolved in the condensation-reagents cocktail (560 μL) and the mixture of DIEA and NMP (560 μL). After shaking for 20 min at 25 °C, the reaction solution was removed and the resin was washed with DMF (2 mL, 5 times), CH_2Cl_2 (2 mL, 3 times) and DMF (2 mL, 3 times). The Fmoc deprotection reactions with piperidine and coupling reactions of Fmoc-protected amino acid were repeated following the designed sequence. After the final Fmoc deprotection reaction and washing, to the resin was added acetic anhydride in CH_2Cl_2 (25%, 2 mL) and the reaction tube was shaken for 10 min at 25 °C. After removal of the reaction solution, the resin was washed with CH_2Cl_2 (2 mL, 3 times), DMF (2 mL, 5 times), CH_2Cl_2 (2 mL, 5 times). To the resin was added the cleavage cocktail (2.5 mL) and the reaction tube was left to stand for 90 min at 25 °C with gentle shaking every 30 min. The solution was collected into a polypropylene centrifuge tube by filtration. The reaction tube was rinsed with TFA (500 μL, 3 times), which is also collected by filtration. To the centrifuge tube was added Et_2O (40 mL) and the tube was mixed on a vortex device for 1 min and centrifuged at 4 °C (3500 × *g*, 5 min), followed by removal of the supernatant liquid. After repeating this process for 3 times, the peptide was dried under vacuum over 2 h at 25 °C, dispersed in water and lyophilized.

MALDI-TOF MS (2,5-dihydroxybenzoic acid, reflector positive): m/z calculated for MBP ($C_{34}H_{50}N_{15}O_{10}^+$) 828.379; found 828.486, Chol-MBP ($C_{60}H_{91}N_{16}O_{15}^+$) 1275.677; found 1275.940.

4. Sample Preparations

1) Bicelles

As a typical procedure for the preparation of DPPC/Chol-MBP bicelles, DPPC (14.8 mg, 20 μmol) and Chol-MBP (6.40 mg, 5.0 μmol) were dissolved in water (0.402 mL). The mixture was heated up to 60 °C and then allowed to cool to 25 °C. This heating-cooling cycle was repeated three times where the resulting solution became transparent. The resulting dispersion was diluted with water to afford DPPC/Chol-MBP bicelles (final concentration = 0.2–1.0 wt%). DPPC/Chol-MBP/Cu bicelles (0.2–1.0 wt%) were prepared by diluting the dispersion of DPPC/Chol-MBP bicelles (5.0 wt%) with an aqueous solution containing $CuCl_2$ (0.0–3.0 equivalents of Cu^{2+} to Chol-MBP). ^{FL}DPPC/Chol-MBP/Cu bicelles were prepared by the same method as that of DPPC/Chol-MBP/Cu bicelles using a mixture of DPPC (14.8 mg, 20 μmol), Chol-MBP (6.40 mg, 4.0 μmol) and Cy5-labeled DPPE (5.30 μg, 4.5 nmol) that were synthesized according to the reported method. DPPC/CHAPSO bicelles were prepared by the same method as DPPC/Chol-MBP bicelles except for using CHAPSO (3.15 mg, 5.0 μmol) instead of Chol-MBP (6.40 mg, 5.0 μmol).

2) ^{FL}DPPC Vesicles

For the preparation of ^{FL}DPPC vesicles, Cy5-DPPE (10 μg, 8.4 nmol) and DPPC (40.0 mg, 54.5 μmol) were dissolved in water (0.76 mL). The mixture was heated up to 60 °C and then cooled down to 25 °C.

5. Cytotoxicity Assay

3T3 cells (3.0×10^3 cells/well) plated onto an 8-well chambered cover glass were incubated in DMEM containing 10% FBS at 37 °C with 5% CO_2 for 24 h. The cell samples were rinsed twice with D-PBS prior to use. 3T3 cells were treated with DPPC/CHAPSO mixture ([DPPC]/[CHAPSO] = 4/1, total content: 1 wt%) or DPPC/Chol-MBP/Cu bicelle ([DPPC]/[Chol-MBP]/[Cu^{2+}] = 4/1/2, total content: 1 wt%) in DMEM, and incubated at 37 °C with 5% CO_2 for 4 h. The 3T3 cell samples were rinsed twice with D-PBS and further incubated at 37 °C for 22 h (26 h-incubation in total) with 5% CO_2 in EMEM containing 10%

FBS. For the cell viability test, the 3T3 cell samples were incubated with Cell Counting Kit-8 reagents (10 μl) for 30 min and subjected to electronic absorption spectroscopy at 450 nm.

6. Blood Circulation

Blood circulation profiles were investigated as reported previously.[1,2] Typically, mice (Balb/c, female, 7 weeks) were injected via tail artery under anesthesia with 100 μL of sample solutions containing ^{FL}DPPC vesicles (5.0 wt%) or ^{FL}DPPC/DPPC/Chol-MBP/Cu bicelles (5.0 wt%), and set for earlobe capillary observation by intravital CLSM (λ_{ext} = 640 nm). The fluorescence intensities at the earlobe vein and skin were monitored continuously.

7. Supplementary Figures

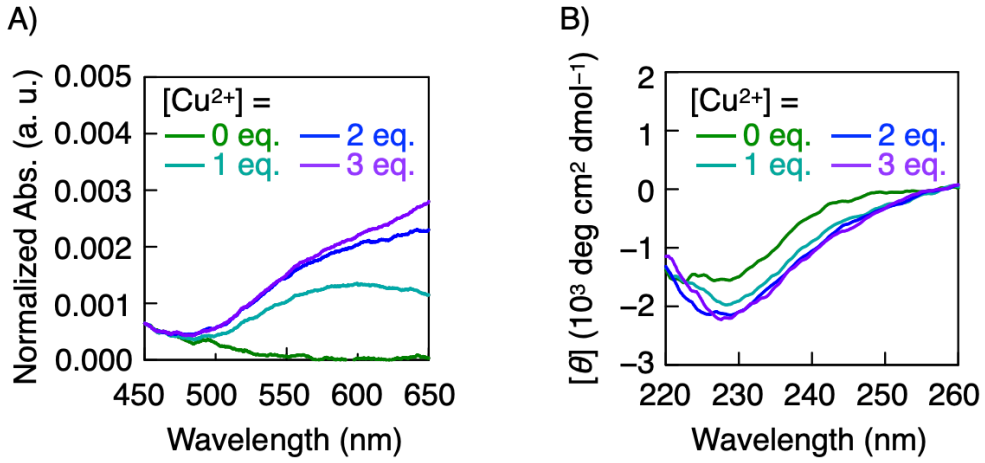

Fig. S1 (A) Absorption and (B) CD spectra of a DPPC and Chol-MBP mixture dispersed in water before and after the addition of 1.0, 2.0, and 3.0 equivalents of Cu^{2+} to MBP ([MBP] = 2.0 mM, [$CuCl_2$] = 0.0–6.0 mM, 25 °C).

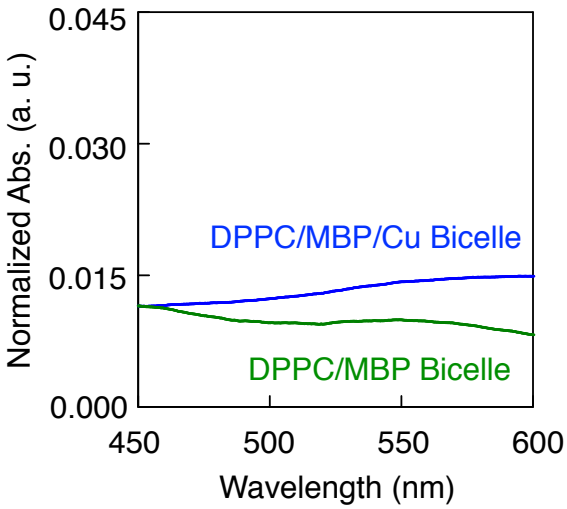

Fig. S2 Absorption spectra of DPPC/MBP bicelle (5.0 wt%) without (green) and with (blue) Cu^{2+} ([DPPC]/[Chol-MBP]/[Cu^{2+}] = 4/1/0 and 4/1/2, respectively).

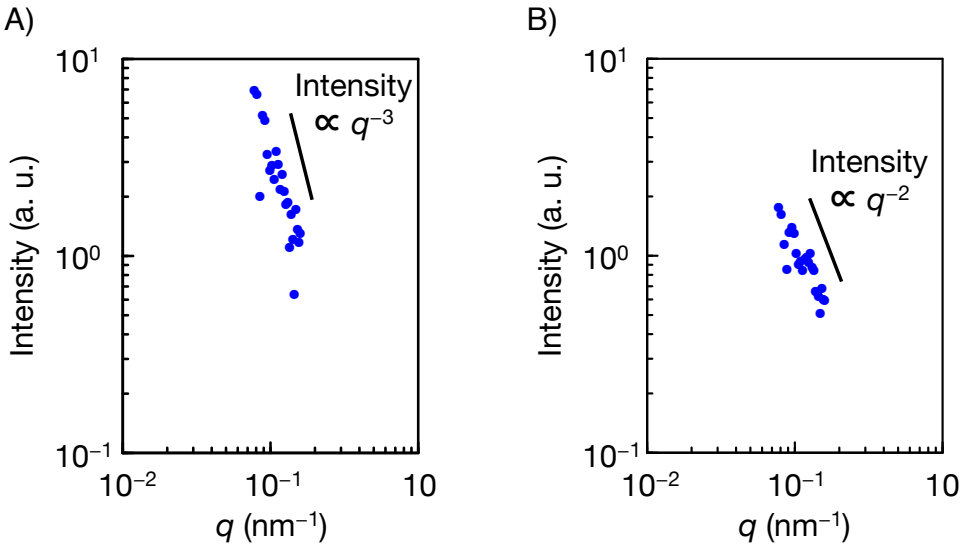

Fig. S3 SAXS profiles of 0.2 wt% mixture of DPPC and Chol-MBP (A) without and (B) with Cu^{2+} ([DPPC]/[Chol-MBP]/[Cu^{2+}] = 4/1/0 and 4/1/2, respectively).

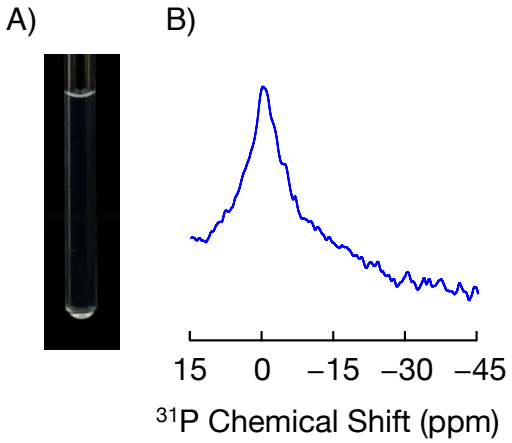

Fig. S4 (A) Photograph and (B) ^{31}P NMR spectrum of the mixture of DPPC, Chol-MBP, and Cu^{2+} ([DPPC]/[Chol-MBP]/[Cu^{2+}] = 4/1/1, total content: 1 wt%, 25 °C) after the incubation with FBS (1 wt%) at 25 °C for 18 h.

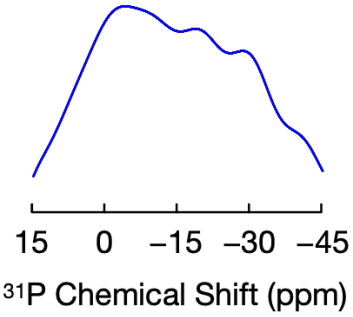

Fig. S5 ^{31}P NMR spectrum of the mixture of DPPC, Chol-MBP, and Cu^{2+} ([DPPC]/[Chol-MBP]/[Cu^{2+}] = 4/1/3, total content: 1 wt%, 25 °C) after the incubation with FBS (1 wt%) at 25 °C for 4 h.

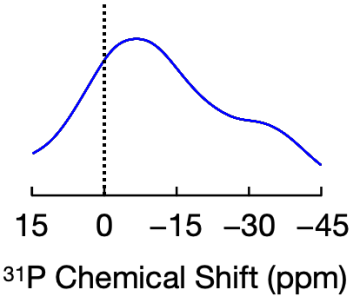

Fig. S6 ^{31}P NMR spectrum of the mixture of DPPC/Chol-MBP/Cu bicelle ([DPPC]/[Chol-MBP]/[Cu^{2+}] = 4/1/2, total content: 0.2 wt%, 25 °C) after the incubation with glutathione (30 μM) at 25 °C for 4 h.

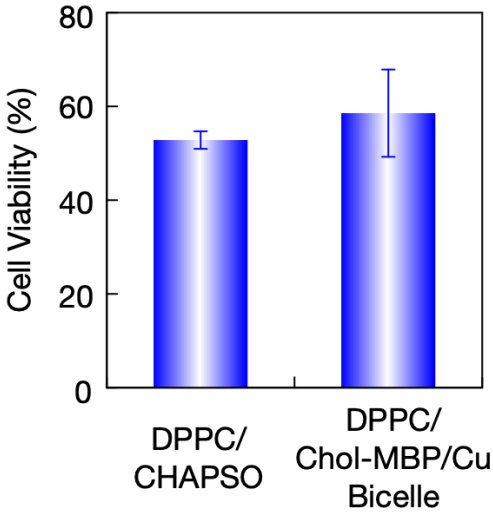

Fig. S7 Cell viability of 3T3 cells after the incubation for 24 h with DPPC/CHAPSO mixture ([DPPC]/[CHAPSO] = 4/1, total content: 1 wt%) and DPPC/Chol-MBP/Cu bicelle ([DPPC]/[Chol-MBP]/[Cu^{2+}] = 4/1/2, total content: 1 wt%).

8. References

1. A. Tao, G. L. Huang, K. Igarashi, T. Hong, S. Liao, F. Stellacci, Y. Matsumoto, T. Yamasoba, K. Kataoka and H. Cabral, *Macromol. Biosci.,* 2020, **20**, 1900161.
2. Y. Matsumoto, T. Nomoto, H. Cabral, Y. Matsumoto, S. Watanabe, R. James Christie, K. Miyata, M. Oba, T. Ogura, Y. Yamasaki, N. Nishiyama, T. Yamasoba and K. Kataoka, *Biomed. Opt. Express,* 2010, **1**, 1209.