Supplementary Information (SI) for ChemComm.

Fabrication of a Self-Assembling Peptide for the Inhibition of Cancer Cell Proliferation by Targeting LAT1

Yuki Koba, Kanta Ogura, Masahiko Nakamoto*, Michiya Matsusaki*

Table of Contents

1. Materials and instruments.

1-1. Materials

The following chemicals were purchased from commercial sources and used as received unless otherwise indicated: *N,N*-dimethylformamide (DMF, CAS RN®: 68-12-2), *N,N*-dimethylformamide anhydrous (DMF anhydrous, CAS RN®: 68-12-2), ninhydrin (CAS RN®: 485-47-2), methanol (CAS RN®: 67-56-1), ethanol(99.5) (CAS RN®: 64-17-5), Piperidine (CAS RN®: 110-89-4), acetonitrile anhydrous (CAS RN®:75-05-8), ultrapure water (CAS RN®: 7732-18-5), trifluoroacetic acid (TFA, CAS RN®:76-05-1), diethyl ether (CAS RN®:60-29-7), trypsin (9002-07-7), and EMEM with phenol red and non-essential amino acids (056-08385) were obtained from Wako (Japan). Fmoc-Lys(Boc)-OH (CAS RN®: 71989-26-9), Fmoc-Leu-OH (CAS RN®: 35661-60-0), Fmoc-Val-OH (CAS RN®: 68858-20-8), Fmoc-D-Phe-OH (CAS RN®: 86123-10-6), and 2-chlorotrityl chloride resin (CAS RN®:934816-82-7) were purchased from

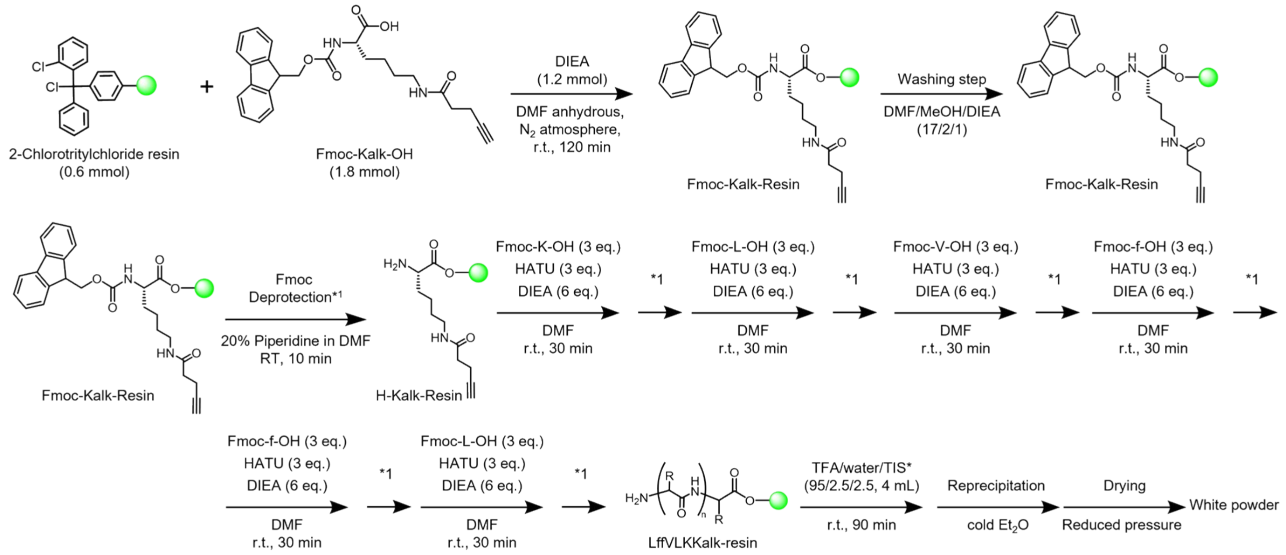
Watanabe Chemical Industries, Ltd. (Japan). 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, CAS RN®:148893-10-1), *N,N*-diisopropylethylamine (DIEA, CAS RN®:7087-68-5), triisopropylsilane (TIS, CAS RN®: 6485-79-6), *tert*-butyl alcohol (CAS RN®:75-65-0), 4-azido-L-phenylalanine (CAS RN®:33173-53-4), L-azidohomoalanine (CAS RN®:120042-14-0), L-azidonorleucine hydrochloride (CAS RN®:1454334-76-9), and Nile red (CAS RN®:7385-67-3) were obtained from Tokyo Chemical Industry (Japan). Acetonitrile (CAS RN®: 75-05-8), cell count reagent sf (07553-44), Dulbecco's modified eagle's medium (DMEM; 08458-45), 1 mol L^{-1} sodium hydroxide solution (CAS RN®: 1310-73-2), 1 mol L^{-1} hydrochloric acid (CAS RN®: 7647-01-0), antibiotics (02892-54), and phosphate buffer saline (D-PBS; D5652-10L) were purchased from Nacalai tesque (Japan). Copper (II) sulfate pentahydrate (CAS RN®: 7758-99-8), sodium L-ascorbate (CAS RN®: 134-03-2), and deuterium oxide (CAS RN®: 7789-20-0) were purchased from Sigma Aldrich (U.S.A.). (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-(pent-4-ynamido)hexanoic acid (CAS RN®: 1159531-18-6) was obtained from Angene International Limited (China). ISOLUTE Si-TMT (CAS RN®:1226494-16-1) was purchased from Biotage Japan (Japan). Natural mica (990065) wasobtained from Nilaco (Japan). Ethylenediaminetetraacetic acid (EDTA; CAS RN®: 10378-23-1) was obtained from Kishida Chemical (Japan). diSulfo-Cy3 azide (BP-23371) was purchased from BROADPHARM (U.S.A.) Eagle's minimum essential medium (EMEM, 30-2003) was obtained from ATCC (U.S.A.). Amino acid uptake assay kit (342-09893) and Cytotoxicity LDH Assay Kit-WST (347-91751) were purchased from Dojindo (Japan). MCF-7 (human breast cancer cell line) was purchased from European Collection of Authenticated Cell Cultures. MDA-MB-231 (human breast cancer cell line), and human embryonic kidney 293 (HEK293) were obtained from KAC Co., Ltd (Japan). Normal human dermal fibroblast (NHDF) was purchased from Lonza (Switzerland). Fetal bovine serum (FBS, Gibco 10270) was obtained from Thermo Fisher Scientific (U.S.A.).

1-2. Instrumentation

Dynamic light scattering was performed using Zetasizer Nano ZS (Malvern, U.S.A). Peptides were purified by Biotage Selekt (Biotage Japan, Japan) equipped with Sfär C18D (Duo 100 Å 30 µm 120 g, Biotage Japan, Japan). ^{1}H NMR spectra were measured using JNM-GSX 400 (JEOL, Japan). HPLC analysis was performed using Prominence series (Shimadzu, Japan) equipped with InertSustain C18 (5 µm, 4.6 × 150 mm) (GL science, Japan). ESI-MS and MALDI-TOF-MS spectra were obtained using JMS-T100LP (JEOL, Japan) and JEOL SpiralTOF JMS-S3000 (JEOL, Japan), respectively. Peptides were sonicated using US-105 (SND, Japan). Fluorescence spectra were recorded using FP-8500 spectrofluorometer (JASCO, Japan). CD spectra were measured by J-1100 (JEOL, Japan). pH of solution was measured by LAQUA twin pH-22B (Horiba, Japan). The AFM images were obtained using SPM-Nanoa (Shimadzu, Japan). The cytotoxicity assay was performed using the multimode micro plate reader Synergy HTX (WakenBtech Co.,Ltd, Japan). Fluorescent images were taken using FV3000 (Olympus, Japan).

2. Synthesis of LffVLKKalk by solid phase peptide synthesis (SPPS).

Scheme S1. Synthetic scheme of LffVLKKalk by SPPS.



LffVLKKalk was prepared by the standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-pahse peptide synthesis on a 0.6 mmol scale (**Scheme S1**). 2-Chlorotrityl chloride resin was applied as a polymeric support. Firstly, the resin was immersed in DMF and stirred for 10 min at room temperature under N_2 bubbling. Then, the mixture (2.88 mL) of (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-(pent-4-ynamido)hexanoic acid (Fmoc-Kalk-OH, 3 eq.) and DIEA (5 eq.) was added into the resin solution. The reaction was carried out for 2 h at room temperature under N_2 atmosphere, then, the resin was collected into a SPPS column. The resin was washed three times with DMF/MeOH/DIEA (17/2/1, 4 mL) mixture. After that, the resin was further washed three times with DMF (4 mL). The Fmoc on the resin was deprotected by 20 vol% piperidine in DMF (8 mL) for 10 min at room temperature. The deprotected resin was washed five times with DMF. Next, Fmoc-protected amino acid-OH (3 eq.) was coupled with the primary amine group on the resin (1 eq.) in presence of HATU (3 eq.) and DIEA in DMF (0.9 M, 6 eq.) for 30 min at room temperature. The proceeding of the reaction was checked by ninhydrin reaction. Briefly, little amount of resin was collected and immersed in ninhydrin in EtOH (0.14 mM, 100 µL). Then, the solution was heated by the heat gun. These synthetic procedures were repeated until the end of peptide sequence. After the deprotection of Fmoc group on Leu, the resin was washed three times with MeOH (4 mL) and was dried in reduced pressure overnight. Then, the deprotection of Boc group and the cleavage of peptide from the resin were performed using the mixture of TFA/MilliQ/TIS (95/2.5/2.5, 6 mL) for 90 min at room temperature. The peptide was precipitated with cold diethyl ether. The precipitate was collected by centrifuge and dried in vacuum at least 2 h. The RP-HPLC (0-40% (0-10 min) and 40-50% (10-40 min) of acetonitrile in water with 0.1% TFA), ^{1}H NMR, and MALDI-TOF-MS of LffVLKKalk ($C_{52}H_{79}N_9O_9$, Mw: 973.60) was carried out (**Figure S1-3**). The HPLC purity was 82%. ^{1}H NMR and MS analysis showed peaks assigned peaks and *m/z* peaks assigned $C_{52}H_{79}N_9O_9Na$, respectively.

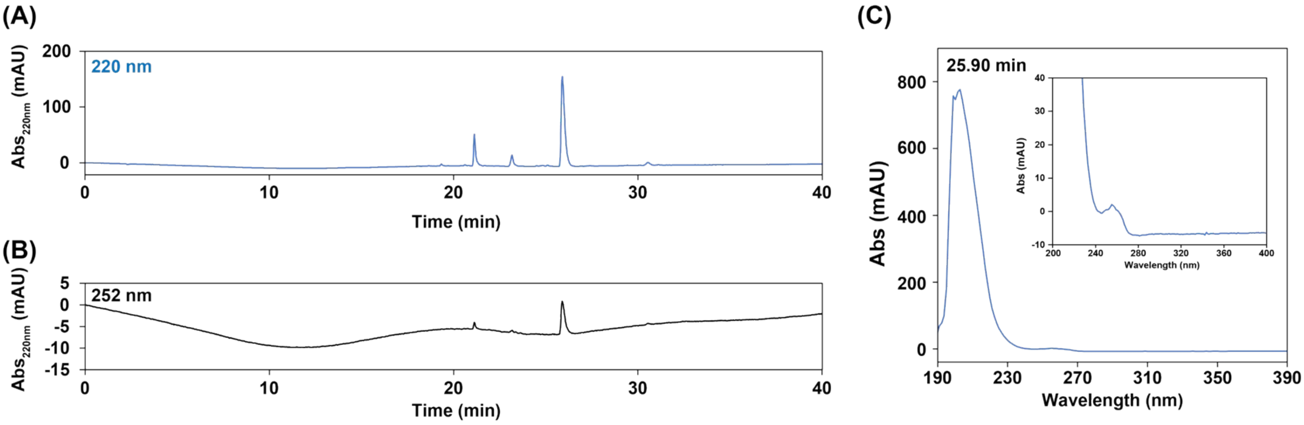


Figure S1. RP-HPLC chromatogram of LffVLKKalk (0.5 mg mL^{-1} in water with 0.1% TFA) monitored by the UV absorbance at (A) 220 and (B) 252 nm. (C) UV-Vis spectra of the elution at 25.90 min. Insert: enlarged view.

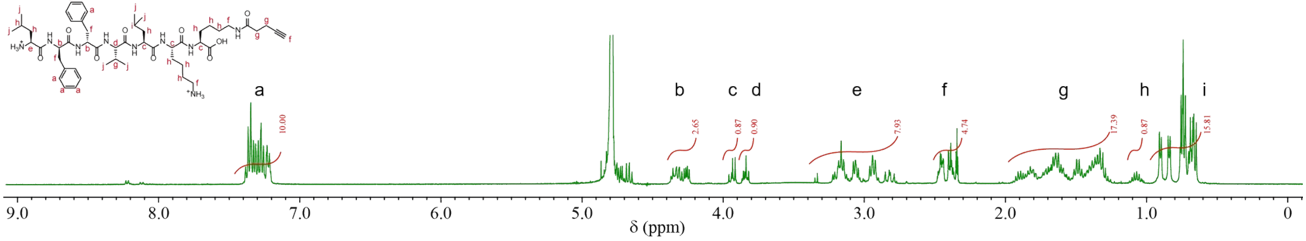
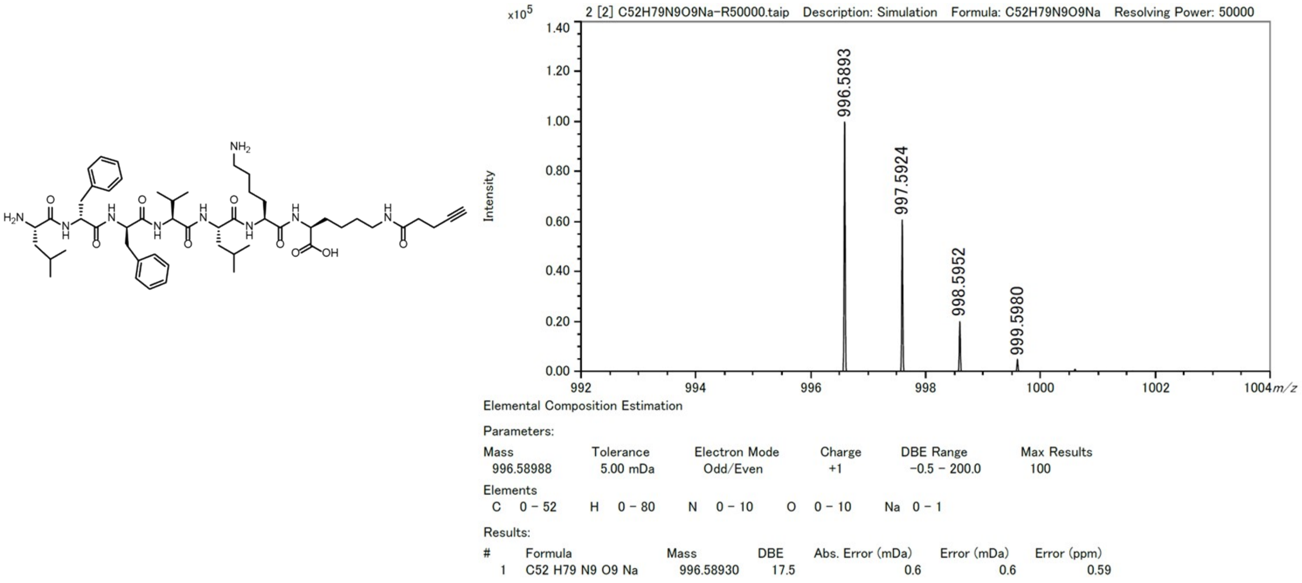


Figure S2. ^1H NMR spectrum of LffVLKKalk in D_2O (400 MHz, 25 °C).

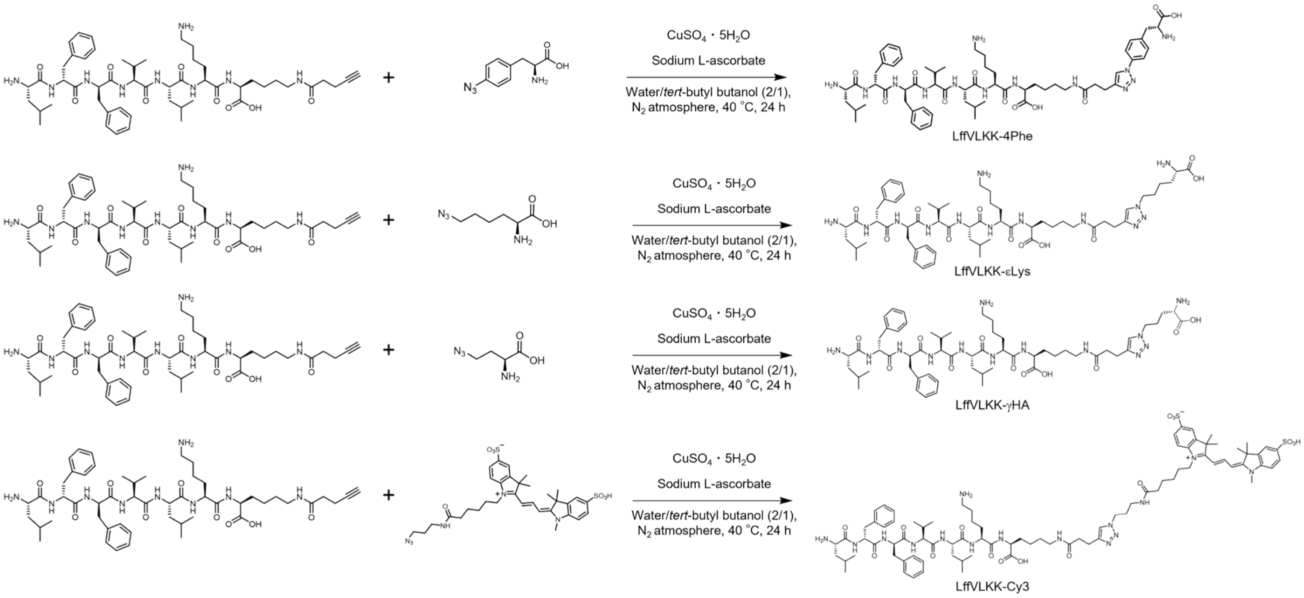


```
Elemental Composition Estimation
Parameters:
Mass        Tolerance   Electron Mode   Charge   DBE Range      Max Results
 996.58988   5.00 mDa    Odd/Even        +1       -0.5 - 200.0   100
Elements
 C   0 - 52    H   0 - 80    N   0 - 10    O   0 - 10    Na   0 - 1
Results:
#   Formula                Mass        DBE    Abs. Error (mDa)   Error (mDa)   Error (ppm)
 1  C52 H79 N9 O9 Na       996.58930   17.5   0.6                0.6           0.59
```

Figure S3. Chemical structure ($C_{52}H_{79}N_9O_9$, m/z = 973.60) and MALDI-TOF MS spectrum of LffVLKKalk.

3. Synthesis of LffVLKK-4Phe, LffVLKK-εLys, LffVLKK-γHA, and LffVLKK-Cy3 by cupper catalyzed azide-alkyne cycloaddition (CuAAC).

Scheme S2. Synthetic scheme of LffVLKK-4Phe, LffVLKK-εLys, LffVLKK-γHA, and LffVLKK-Cy3 by CuAAC.



CuAAC reaction was carried out to obtain LffVLKK-4Phe, LffVLKK-εLys, LffVLKK-γHA, and LffVLKK-Cy3 (**Scheme S2**). First, LffVLKKalk (50 mg, 1 eq.) was dissolved in MilliQ (35 mL). Then, azide compound aq. (1.0 mg mL^{-1}, 1 eq.) and $CuSO_4 \cdot 5H_2O$ aq. (100 mM, 0.5 eq.) was added. After that, *tert*-butyl butanol (17 mL) was also added to peptide solution. The mixture was stirred for 10 min at 40 °C under N_2 bubbling. Then, sodium L-ascorbate aq. (10 mM, 2.5 eq.) was added to the peptide mixture. The reaction was performed for 24 h at 40 °C under N_2 flow. After reaction, ISOLUTE Si-TMT (5 eq.) was applied as a metal scavenger for 30 min, then particles were removed by filtration (220 μm). The solvent was evaporated and the peptide was purified by RP-Flash chromatography. The proceeding of the reaction was confirmed by ^{1}H NMR analysis and MS analysis. In all cases, a peak assigned as the proton of triazole group was confirmed (**Figure S4-S7**). In addition to ^{1}H NMR spectra, MS spectra also exhibited *m/z* from single compound in each product (**Figure S8-S11**), suggesting that successful synthesis of peptides of interest.

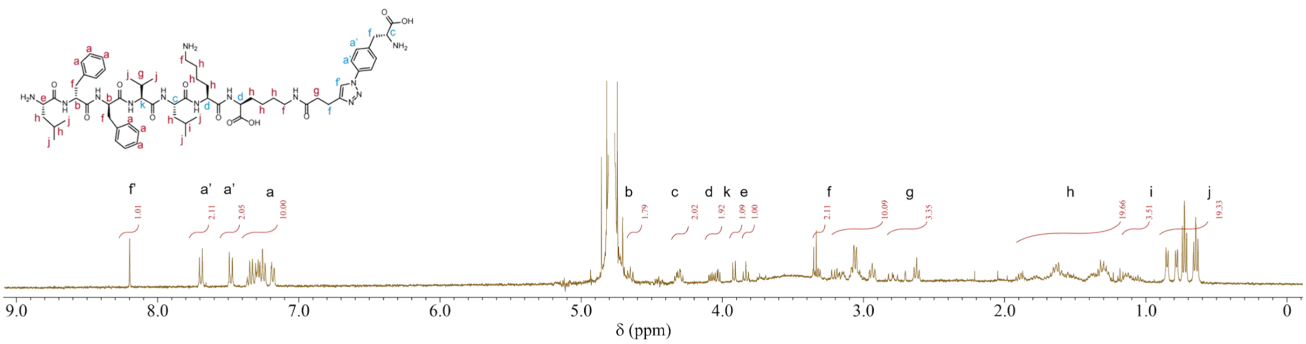


Figure S4. ^{1}H NMR spectrum of LffVLKK-4Phe in D_2O (400 MHz, 25 °C).

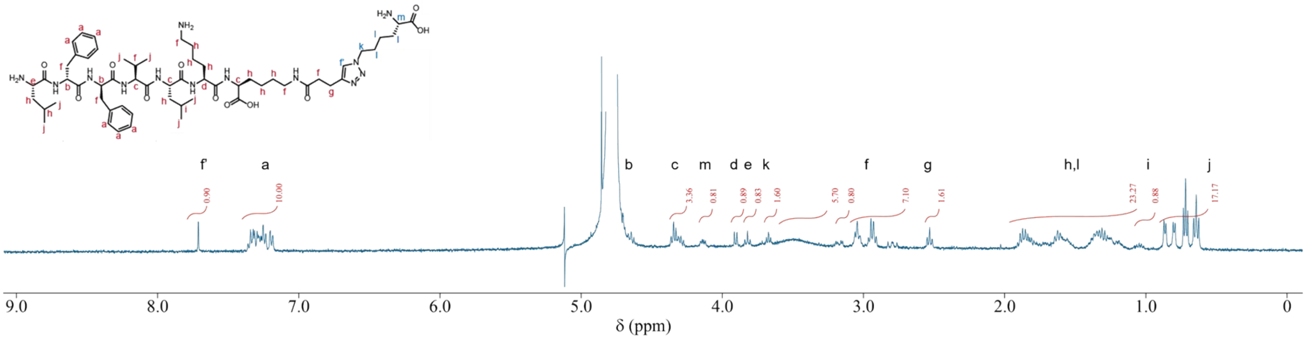


Figure S5. ^{1}H NMR spectrum of LffVLKK-εLsy in D_2O (400 MHz, 25 °C).

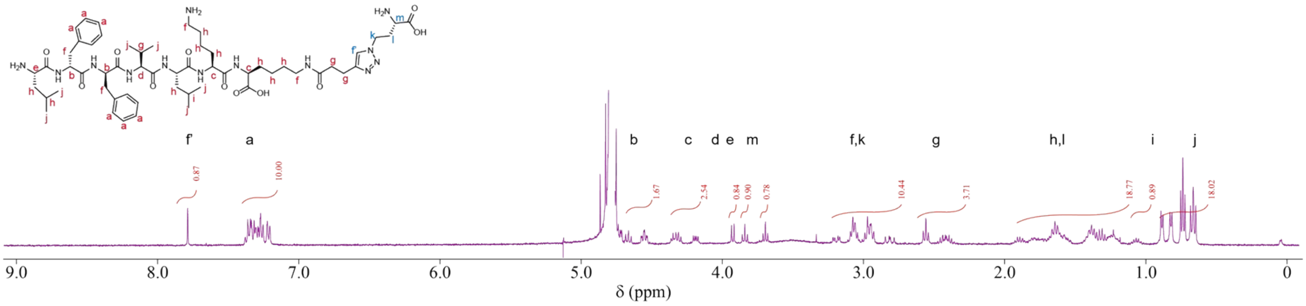


Figure S6. ^{1}H NMR spectrum of LffVLKK-γHA in D_2O (400 MHz, 25 °C).

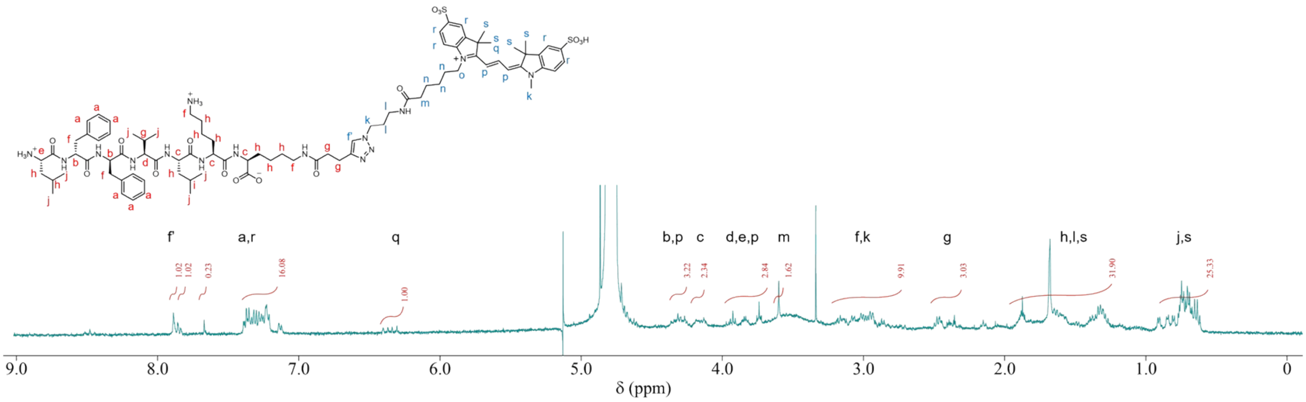
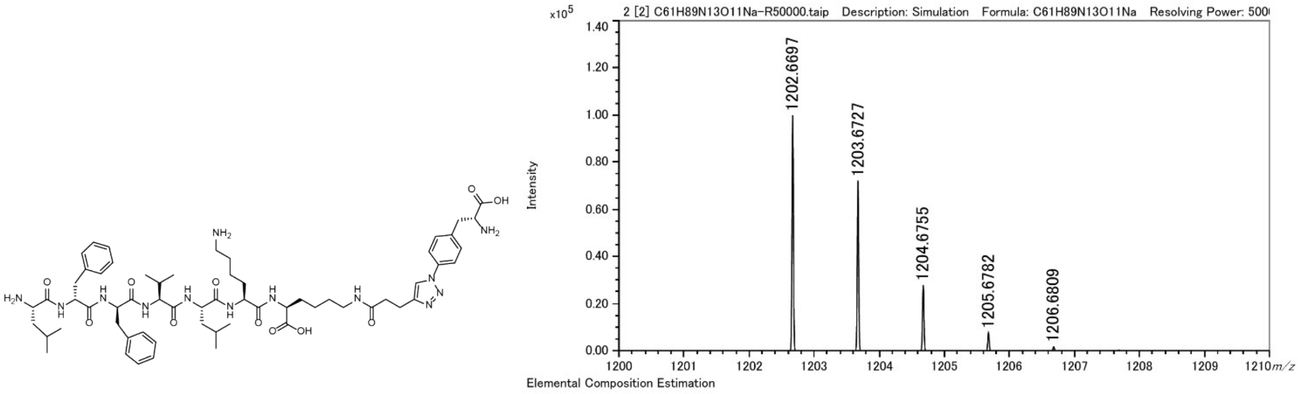


Figure S7. ^{1}H NMR spectrum of LffVLKK-Cy3 in D_2O (400 MHz, 25 °C)

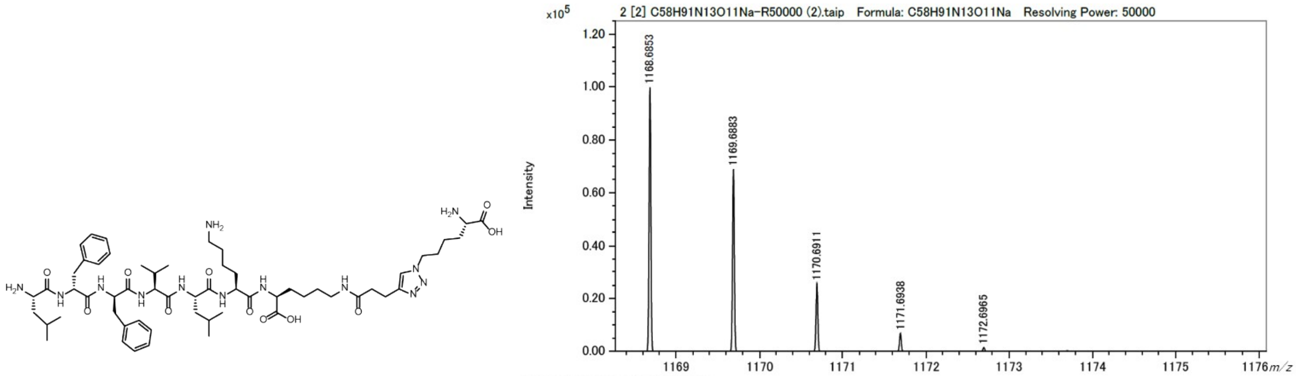


```
Elemental Composition Estimation

Parameters:
Mass          Tolerance     Electron Mode    Charge    DBE Range      Max Results
 1202.67063    5.00 mDa      Odd/Even          +1        −0.5 − 200.0    100
Elements
 C   0 − 61     H   0 − 90     N   0 − 15     O   0 − 12     Na  0 − 1

Results:
#     Formula                 Mass         DBE     Abs. Error (mDa)    Error (mDa)    Error (ppm)
 1    C61 H89 N13 O11 Na      1202.66967   23.5               1.0            1.0           0.80
```

Figure S8. Chemical structure ($C_{61}H_{89}N_{13}O_{11}$, *m/z* = 1179.68) and MALDI-TOF MS spectrum of LffVLKK-4Phe.

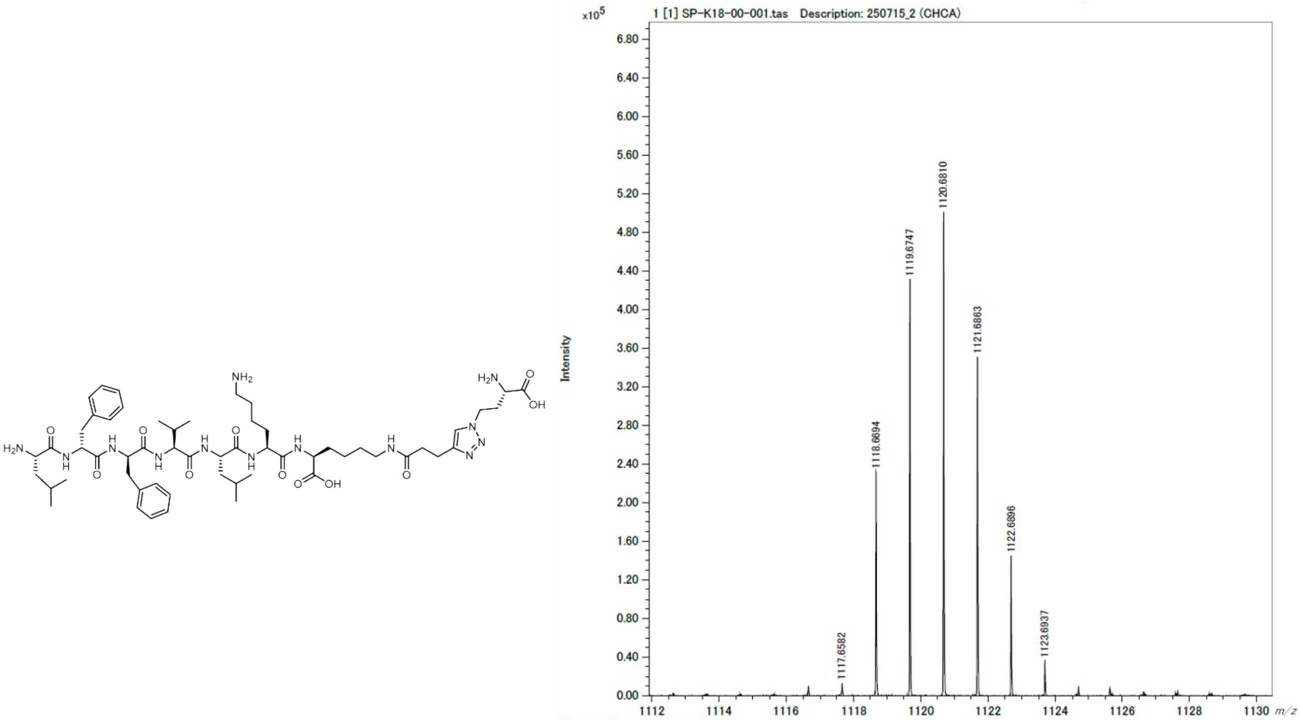


```
Elemental Composition Estimation

Parameters:
Mass          Tolerance     Electron Mode    Charge    DBE Range      Max Results
 1168.68358    5.00 mDa      Odd/Even          +1        −0.5 − 200.0    100
Elements
 C   0 − 60     H   0 − 200     N   0 − 13     O   0 − 11     Na  1 − 1

Results:
#     Formula                 Mass         DBE     Abs. Error (mDa)    Error (mDa)    Error (ppm)
 1    C58 H91 N13 O11 Na      1168.68532   19.5               1.7           −1.7          −1.49
```

Figure S9. Chemical structure ($C_{58}H_{91}N_{13}O_{11}$, *m/z* = 1145.70) and MALDI-TOF MS spectrum of LffVLKK-εLys.



Elemental Composition Estimation

Parameters:

Mass	Tolerance	Electron Mode	Charge	DBE Range	Max Results
1118.66941	5.00 mDa	Odd/Even	+1	−0.5 – 200.0	100

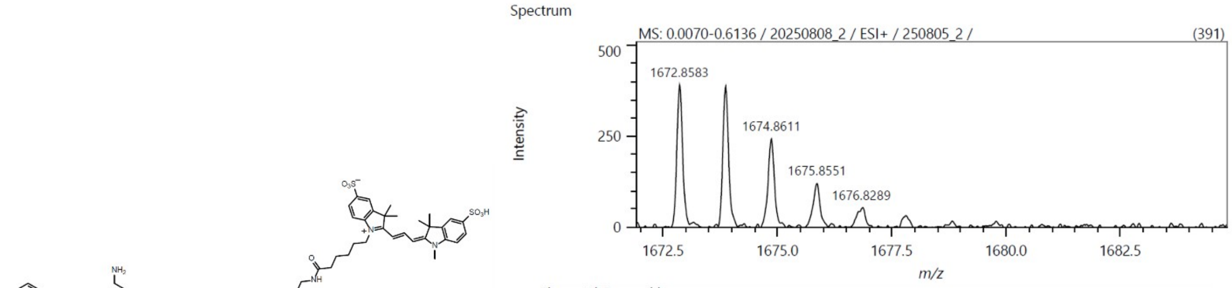
Elements

C 0 – 56 H 0 – 200 N 0 – 13 O 0 – 11 Na 0 – 0

Results:

#	Formula	Mass	DBE	Abs. Error (mDa)	Error (mDa)	Error (ppm)
1	C56 H88 N13 O11	1118.67208	19.5	2.7	−2.7	−2.39

Figure S10. Chemical structure ($C_{56}H_{87}N_{13}O_{11}$, *m/z* = 1117.66) and MALDI-TOF MS spectrum of LffVLKK-γHA.



Elemental Composition

Parameters	
Tolerance:	5.00 mDa
Electron:	Odd/Even
Charge:	+1
DBE:	-2.0 - 80.0

Elements Set 2:

Symbol	C	H	N	O	S	Na
Min	0	0	0	0	2	0
Max	90	150	15	16	2	0

Results

Mass	Intensity	Formula	Calculated Mass	Mass Difference [mDa]	Mass Difference [ppm]	DBE
1672.85829	391.25	C89 H120 N14 O14 S2	1672.85444	3.85	2.30	37.0
		C85 H122 N15 O16 S2	1672.86299	-4.70	-2.81	32.5

Figure S11. Chemical structure ($C_{85}H_{122}N_{15}O_{16}S_2^+$, *m/z* = 1672.86) and ESI MS spectrum of LffVLKK-Cy3.

4. Evaluation of self-assembly ability of LffVLKK-4Phe by DLS, CD measurement, fluorescence imaging, and Nile red staining.

4-1. DLS measurement

Peptides were dissolved in D-PBS adjusted pH to 6.8 at various concentrations and sonicated for 10 min.

Then, DLS measurement was performed using ZEN40 cells (solution volume: 50 µL) under 37 °C. The critical aggregation concentration was calculated using derived count rate. The CSC value was determined by piecewise fitting model as follows[25]:

$$y = a_1 + k_1 x \qquad (x < x_i)$$

$$y = y_i + k_2(x - x_i) \qquad (x \geq x_i)$$

$$y = a_1 + k_1 x_1$$

where y is derived count rate, a_1 is the intercept, x_i is the intersection which is defined as CSC, and y_i is derived count rate at the CSC.

Furthermore, in autocorrelation functions ($g^{(2)}(\tau)$-1), the increase of slow mode concentration from 0.05 to 0.5 mg mL^{-1} (**Figure S12B-G**). In addition, the decrease of initial value of autocorrelation functions ($g^{(2)}(\tau)$-1) was observed above 1.0 mg mL^{-1} (**Figure S12B-G**), suggesting that the decrease of mobility of LffVLKK-4Phe due to the concentration-dependent self-assembly.

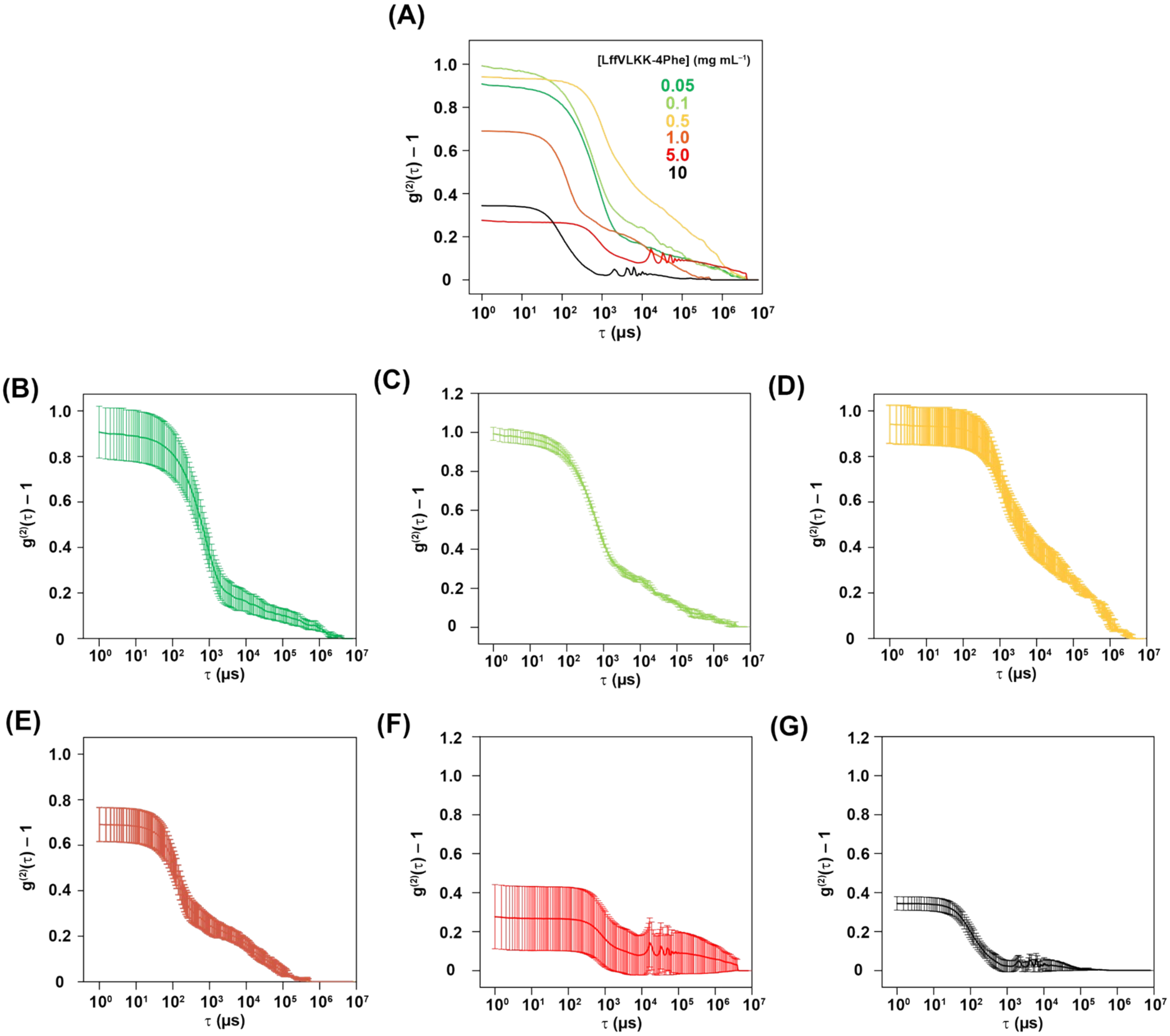


Figure S12. (A) The average values of the autocorrelation function of LffVLKK-4Phe at concentrations of 0.05 (green), 0.1 (light green), 0.5 (yellow), 1.0 (orange), 5.0 (red), and 10 (black) mg mL^{-1}. (B-G) The autocorrelation function of LffVLKK-4Phe with SD at the concentrations of (B) 0.05, (C) 0.1, (D) 0.5, (E) 1.0, (F) 5.0, and (G) 10 mg mL^{-1}.

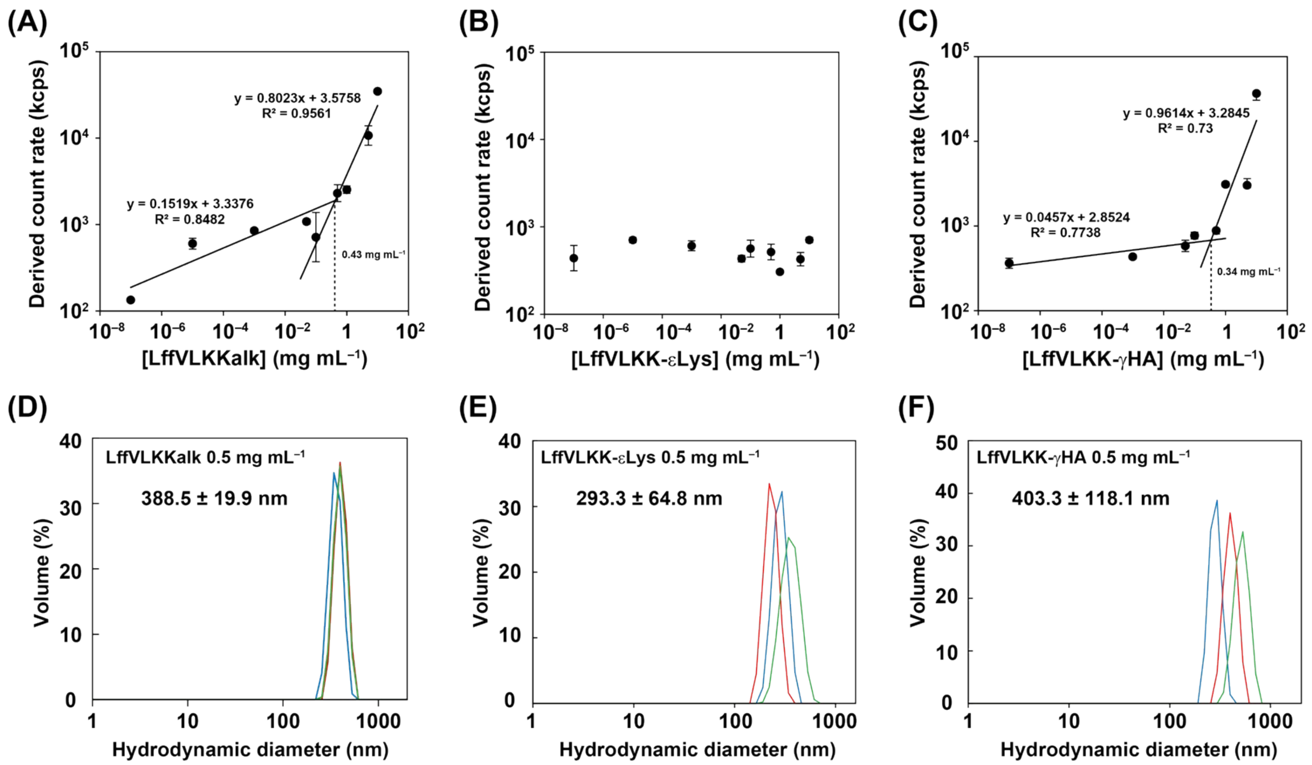


Figure S13. (A-C) Derived count rate of (D) LffVLKKalk, (E) LffVLKK-εLys, and (F) LffVLKK-γHA at various concentrations in D-PBS (pH 6.8). (D-E) Volume-based hydrodynamic diameter distribution of (D) LffVLKKalk, (E) LffVLKK-εLys, and (F) LffVLKK-γHA at 0.5 mg mL^{-1}.

4-2. CD measurement

LffVLKK-4Phe was dissolved in D-PBS adjusted pH to 6.8 at various concentrations and sonicated for 10 min. Then, CD spectra were obtained using 0.1 mm cuvette (solution volume: 40 μL) under 37 °C. In the case of 5.0 mg mL^{-1}, CD spectrum of the supernatant was obtained because the precipitation was confirmed (**Figure S13**).

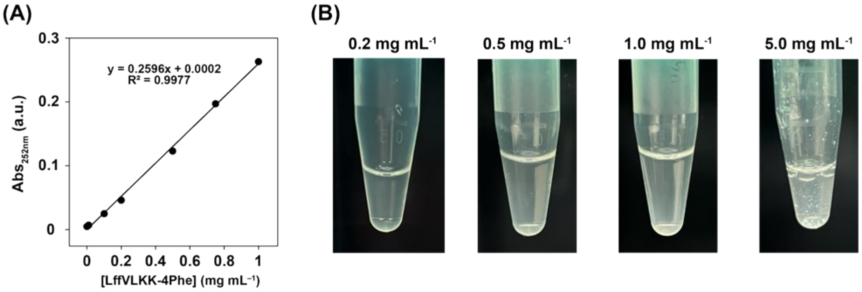


Figure S14. (A) The calibration curve of LffVLKK-4Phe in PBS at pH 6.8. (B) Pictures of LffVLKK-4Phe solution in D-PBS (pH 6.8) at various concentrations.

4-3. AFM observation

Peptides were dissolved in filtered 15-times dilution of D-PBS (pH 6.8), then sonicated for 10 min. Peptide solution (50 μL) was dropped to the freshly cleaved mica and dried overnight in 6 well plate. Then, mica substrate was washed once with MilliQ (50 μL) using pipette and then N_2 flow. The substrates were stored in sealed containers until observation. The morphologies of nanostructures on mica were imaged using an SPM-Nanoa (Shimadzu, Japan) with a multipurpose XYZ scanner (10 μm) in contact mode. Silicon SPM-sensor (POINTPROBE CONT-10) were used.

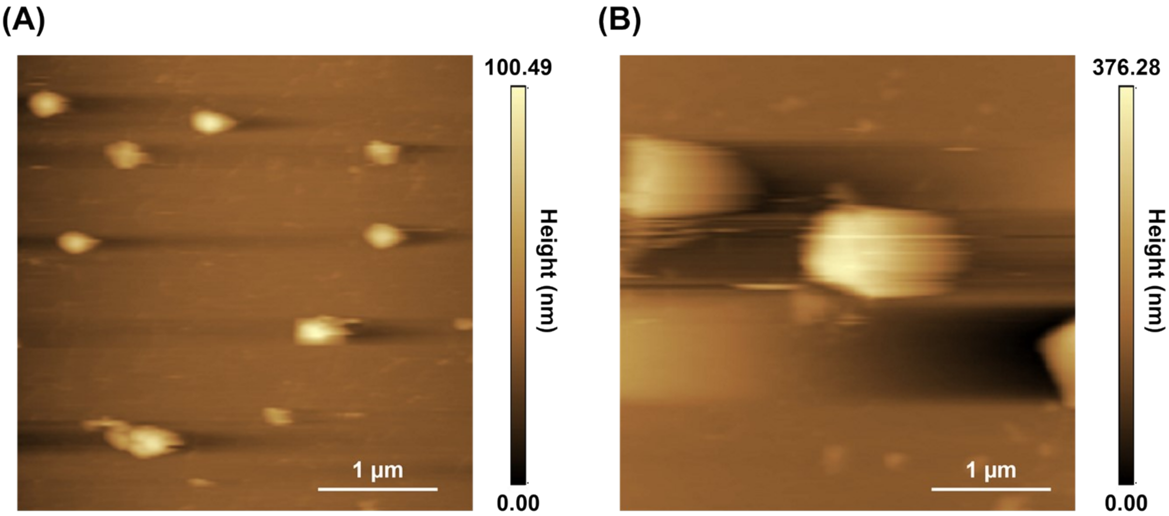


Figure S15. AFM image of LffVLKK-4Phe prepared at (A) 0.25 and (B) 0.5 mg mL^{-1} in 15-times diluted D-PBS.

4-4. Fluorescence observation

LffVLKK-4Phe was dissolved in D-PBS adjusted pH to 6.8 at various concentrations (0.2, 0.5, and 1.0 mg mL^{-1})and sonicated for 10 min. LffVLKK-Cy3 was dissolved in D-PBS adjusted pH to 6.8 at 100 μM. Then, LffVLKK-4Phe solution (99 μL) was mixed with LffVLKK-Cy3 (1 μL) and vortexed. 50 μL of mixture was dropped on glass slide and covered with cover glass. Assemblies of LffVLKK-4Phe were expected to be stained by LffVLKK-Cy3 because they have same motif for self-assembly. Fluorescence imaging was performed by FV3000 with following setting: a 633 nm argon laser (3.5%) for the excitation with emission collected by Cy3 filter (detector voltage 500 V) for Cy3. All images were taken using the same settings. Fluorescence microscopy revealed that micro-scale assemblies at concentrations above 0.5 mg mL^{-1} (**Figure S16**), suggesting that self-assembly of LffVLKK-4Phe in response to the increase of concentration.

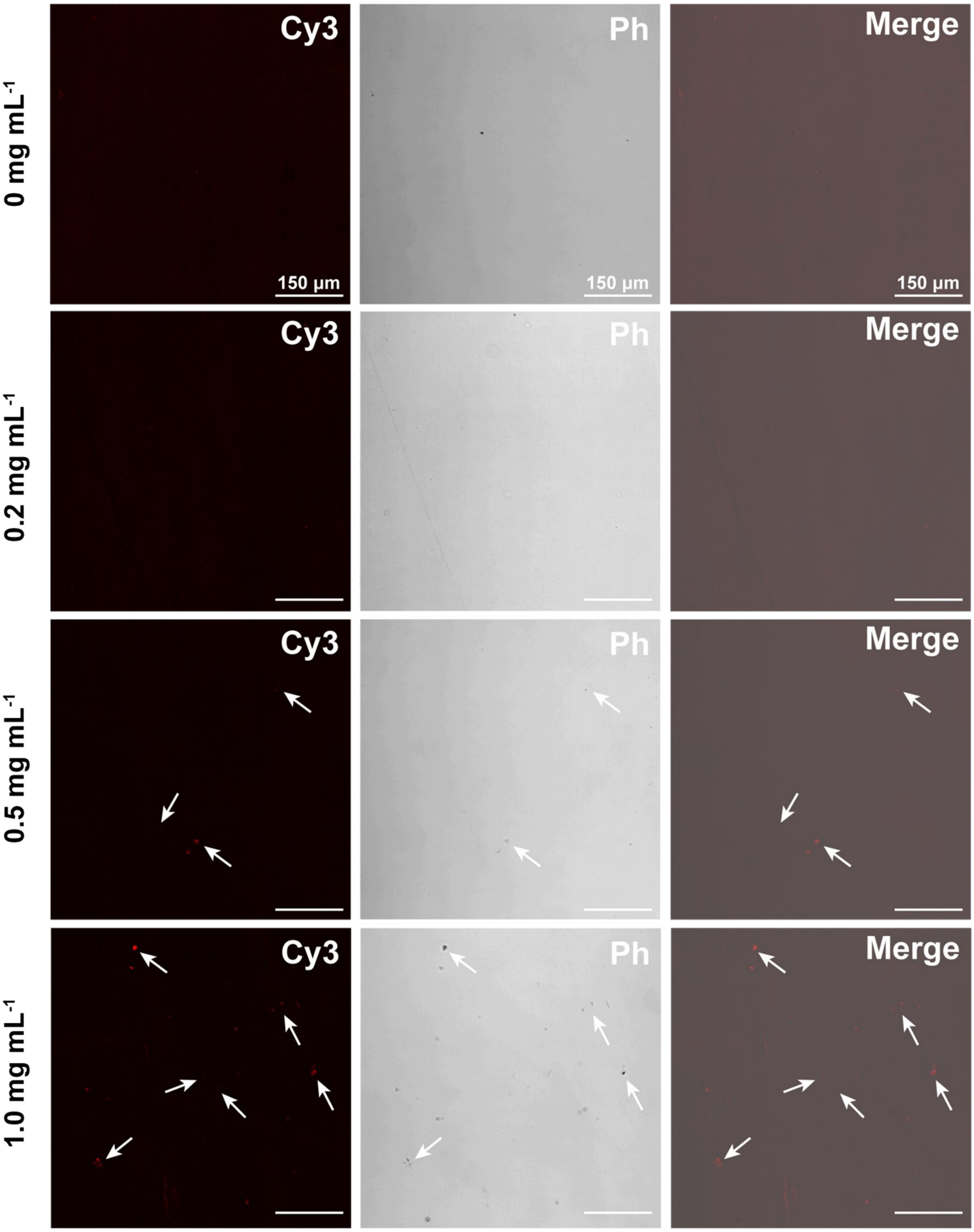


Figure S16. Fluorescence and phase-contrast images of the mixture LffVLKK-4Phe with LffVLKK-Cy3 in D-PBS (pH

6.8). Arrows mean assemblies of LffVLKK-4Phe. Some assemblies were observed by fluorescence microscopy but not in phase-contrast microscopy.

4-5. Nile red staining

LffVLKK-4Phe solution (999 μL, 0.5 mg mL^{-1} in D-PBS (pH 6.8)) was prepared, then Nile red (1 μL, 1 mM in acetonitrile) was added. After the incubation for 30 min in the dark, the fluorescence spectrum was measured with excitation 553 nm. LffVLKK-4Phe solution exhibited a higher Nile Red fluorescence intensity than the peptide-free solution (**Figure S17**), indicating that hydrophobic space emerged by self-assembly of LffVLKK-4Phe.

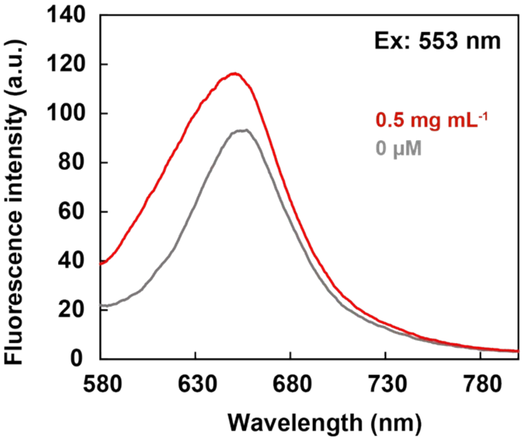


Figure S17. Fluorescence spectra of the mixture of LffVLKK-4Phe in D-PBS (pH 6.8) at the concentrations of 0 and 0.5mg mL^{-1} with Nile red (2 μM).

5. Computational simulation of self-assembly of LffVLKK-4Phe and binding affinity of LffVLKK-4Phe to LAT1.

5-1. MD simulation of self-assembly of LffVLKK-4Phe

The three-dimensional structure of LffVLKK-4Phe for MD simulation was prepared by Chem3D software (25.5.0). The size of LffVLKK-4Phe was calculated as 2.07 nm by measuring the distance between N atom on the Lys residue and terminal O atom on the LAT1 ligand after MM2 energy minimization in the software.

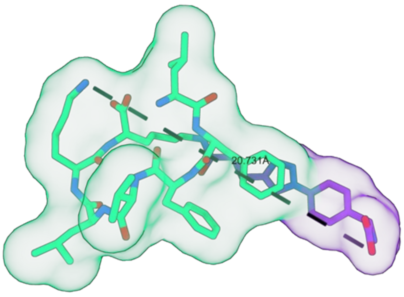


Figure S18. The structure of LffVLKK-4Phe after MM2 energy minimization. Dot black line means measured distance.

All the simulations were performed in GROMACS version 2025.1 using the amber99sb force field and a temperature of 300 K together with a 2 fs timestep. For 100 ns simulation, six and forty peptide molecules were placed into a cubic box with an edge length of 80 Å, for 100 ns and 1 µs simulation, respectively. Systems subsequently were solvated with tip3 water molecules. Then, 100 mmol of Na^+ and Cl^- ions were added to the systems. Firstly, systems were energy minimized using steepest descent minimization until the maximum force was less than 100 kJ mol^{-1} nm^{-1}. After energy minimization, the systems were equilibrated under NVT conditions using the V-rescale thermostat, then were further equilibrated under NPT conditions and 1 bar using the V-rescale thermostat and the C-rescale barostat. After NPT equilibration, MD simulation was performed.

5-2. Docking simulation of biding affinity of LffVLKK-4Phe to LAT1

Docking simulations were performed with the Autodock Vina. A structure of LAT1 obtained from cryo-EM (PDB ID: 8kdn) and LffVLKK-4Phe or Phe were utilized as a receptor and ligands, respectively. In simulations, grid box included full-length of protein and five residues (T62, S66, F252, Y259, N404) were selected as flexible residues. The exhaustiveness was 500 for each ligand.

6. Cell culture

6-1. Cell culture

MCF-7 was cultured in EMEM supplemented with 10% FBS and 1% antibiotics. MDA-MB-231, NHDF, and HEK293 were cultured in DMEM supplemented with 10% FBS and 1% antibiotics. For culture under hypoxic conditions, cells were incubated at 37 °C under an atmosphere of 1% O_2, 5% CO_2, 94% N_2 in a humidified incubator. For culture under normoxic conditions, cells were grown in a humidified incubator at 37 °C under 5% CO_2.

6-2. WST-8 assay

MCF-7 and MDA-MB-231 (5.0×10^3 cell/well) were seeded onto 96-well plates using culture medium adjusted to pH 6.8 by 1 M HCl aq. and incubated for 24 h under 1% O_2 (hypoxic conditions). After incubation, cells were treated with peptides and Phe(4-N_3) incubated for 72 h under hypoxic conditions. Then, plates were washed with D-PBS and added cell counting kit-8 solution diluted 10 times by culture medium. After 3 h, plates were diluted 2 times by D-PBS and centrifuged (4000 rpm, 5 min). 80 µL supernatant were collected to 96-well plates and measured the absorbance at 450 nm using a micro plate reader. In addition, NHDF (2.0×10^3 cell/well), and/or HEK293 (2.0×10^4 cell/well) were seeded onto 96-well plates using DMEM (pH 7.4) and incubated for 24 h under 20% O_2 (normaxic conditions). After incubation, cells were treated with LffVLKK-4Phe and incubated for 72 h under normoxic conditions. Then, the same procedure as mentioned above was performed to reveal the cytotoxicity of LffVLKK-4Phe on healthy cells.

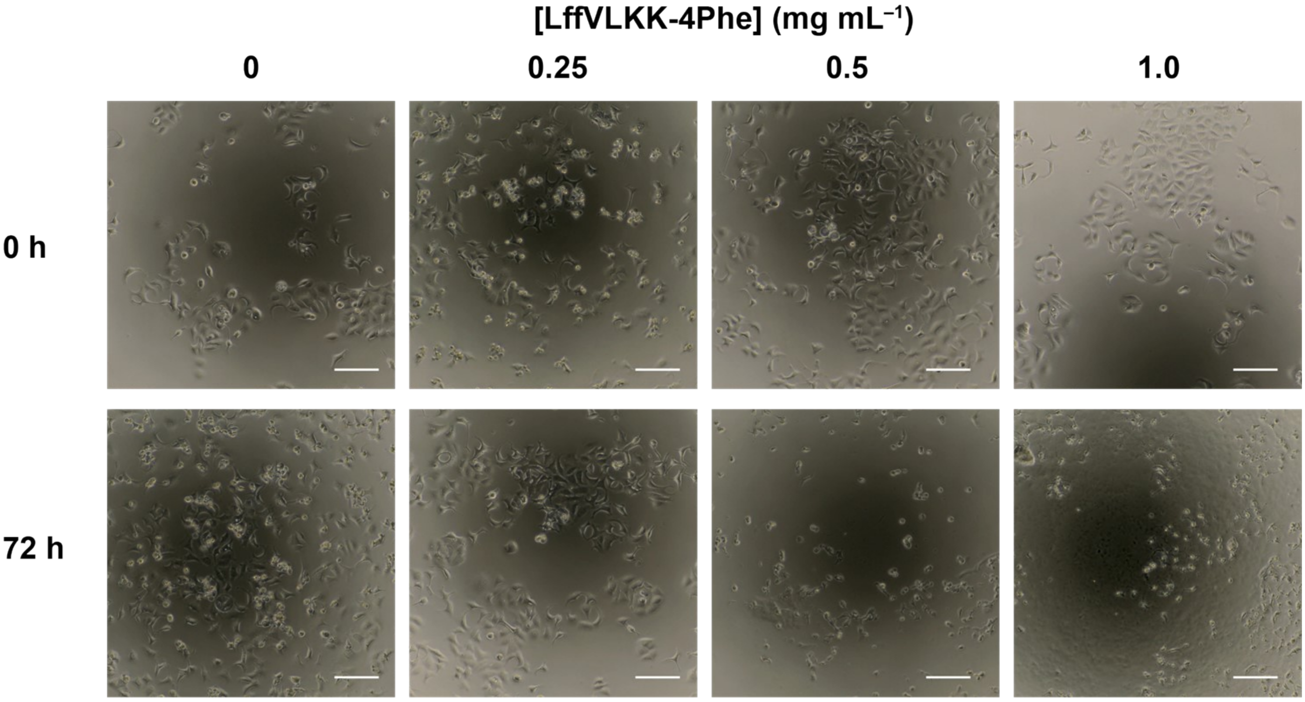


Figure S19. Phase-contrast images of MCF-7 treated with LffVLKK-4Phe for 0 and 72 h. Scale bar shows 150 µm.

6-3. Amino acid uptake assay

MCF-7 and MDA-MB-231 (3.0×10^4 cell/well) were seeded onto 96-well glass-bottom plates using culture medium adjusted to pH 6.8 and incubated for 24 h under hypoxic conditions. Then, amino acid uptake assay was performed by following official protocol of the assay kit. Briefly, cells were washed three times with HHBS and treated p-Boronophenylalanine (BPA) uptake solution with and without LffVLKK-4Phe for 30 min at normoxic conditions. Then, cells were washed three times with HBSS and treated with working solution for 5 min at room temperature. The fluorescence was recorded by microplate reader.

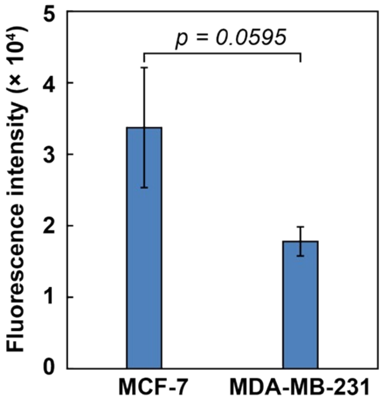


Figure S20. p-Boronophenylalanine (BPA) uptake capacity in MCF-7 and MDA-MB-231. Statistical significance was determined using one-way ANOVA followed by Tukey's post-test (n = 3). Data are mean ± SD.

6-4. LDH assay

MCF-7 (5.0×10^3 cell/well, 200 µL) was seeded onto 96-well plates using culture medium adjusted to pH 6.8 and incubated for 24 h under hypoxic conditions. After incubation, cells were treated with LffVLKK-4Phe (0.5 and 1.0 mg mL^{-1}, 100 µL) incubated in EMEM without pyruvate for 30 min under normaxic conditions. Then, EMEM without pyruvate (100 µL) was added. Supernatant was collected to another 96 well plate, and 10 mM NaOH aq. (2 µL) was added. After that, working solution was (100 µL) added into the well and incubated for 30 min at room temperature in the dark. Stop solution (50 µL) was added and the absorbance at 490 nm was measured by microplate reader For positive control, cells were treated with lysis solution for 30 min instead of LffVLKK-4Phe treatment. For negative control, cells were treated with EMEM without pyruvate and peptide.

LDH release (%) was calculated following equation:

$$LDH\ release\ (\%) = \frac{Abs._{treatment} - Abs._{negative}}{Abs._{positive} - Abs._{negative}} \times 100$$

where Abs.$_{\text{treatment}}$ is the absorbance of samples treated with LffVLKK-4Phe, Abs.$_{\text{negative}}$ is the absorbance of samples without treatment, and Abs.$_{\text{negative}}$ is the absorbance of samples treated with lysis buffer.