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FULL PAPER

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Short self-assembling peptides with a urea bond: A new type of supramolecular peptide hydrogel materials

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Abstract

There is an increasing need to develop short self-assembling peptides (SAPs) that can form hydrogels for cell engineering and biomedical applications. In this study, we proposed new short self-assembling peptides with a symmetric structure via a urea bond. $(FFiO)_2$ and $(FFiK)_2$ were designed as amphiphilic peptides with a hydrophobic domain inside of zwitterionic hydrophilic ends and a urea bond embedded in the hydrophobic domain. The two peptides were synthesized by a liquid-phase method. The simple structural design of these peptides makes their synthesis on a large scale possible. Both $(FFiO)_2$ and $(FFiK)_2$ assembled into β-sheet structure and formed stable hydrogels under physiological pH conditions in a pH-responsive manner. The urea bond was important for the formation of transparent hydrogels. Since $(FFiK)_2$ exhibited self-assembling properties superior to those of $(FFiO)_2$, $(FFiK)_2$ hydrogels were used as scaffolds for cell culture. $(FFiK)_2$ hydrogels supported cell proliferation without significant cytotoxicity. Therefore, $(FFiK)_2$ is a beneficial supramolecular peptide hydrogelator for cell engineering.

KEYWORDS

amphiphilic peptide, cell culture, hydrogel, self-assembling peptide, urea bond

1 | INTRODUCTION

The self-assembly of peptides has received much attention over the last two decades, because precisely designed peptides can assemble into various nanostructures such as fibers, sheets and spherical micelles. In particular, peptide nanofibers that can form hydrogels have been extensively developed for tissue engineering and biomedical applications.[1] There are many design approaches for self-assembling peptides (SAPs) based on amphiphilic β-sheet or α-helical structures and a peptide amphiphile.[2,3] The first SAP to be designed was EAK16 (Ac-AEAEAKAKAEAEAKAK-NH_2), an amphiphilic 16-mer β-sheet peptide.[4] The structure-assembling property relationships of amphiphilic peptides designed based on the EAK16 peptide have been investigated,[5–7] and shorter SAPs, such as Ac-$(FKFE)_2$-NH_2, were discovered.[8,9] Compared to longer peptides, short peptides have advantages in that their synthesis requires fewer steps and their purification is easier.

Recently, simple amino acid derivatives and dipeptides have been recognized as supramolecular hydrogelators.[10–12] The first such example is dibenzoyl-L-cystine, which can form a self-supporting hydrogel at a 0.1 wt% concentration.[13] After it was reported that a diphenylalanine (FF) peptide self-assembled into nanotubes in aqueous medium,[14] FF derivatives with an aromatic cap, such as Fmoc,[15] at their N-terminus were reported as small molecular SAPs.[12] In addition, non-natural phenylalanine derivatives, such as 4-nitrophenylalanine, are also used in low-molecular-weight hydrogelators.[16] Small molecular SAPs are easier to synthesize than traditional amphiphilic SAPs. However, special care is required for the combination of aromatic amino acids with aromatic or hydrophobic cap groups to obtain transparent hydrogels,[17,18] because strong π-π stacking interactions often cause SAPs to crystalize.

In this study, we designed new short self-assembling peptides, $(FFiX)_2$, with a symmetric structure via a urea bond (Figure 1A). Due to their bolaamphiphilic form, $(FFiX)_2$ was easy to synthesize on a

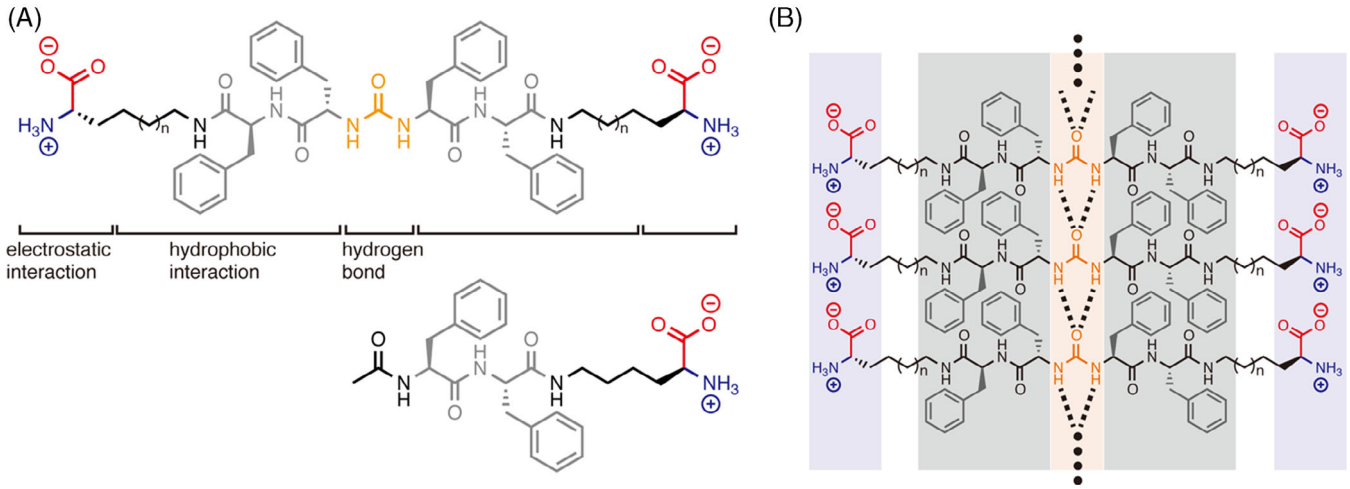


FIGURE 1 A, Molecular design of $(FFiO)_2$, $(FFiK)_2$ and Ac-FFiK. B, Schematic illustration of self-assembly of $(FFiX)_2$

large scale, and a hydrophobic/aromatic domain placed inside the hydrophilic ends suppressed the crystal packing of the peptides. In addition, the urea bond embedded in the hydrophobic domain is expected to form a strong hydrogen bond network between peptides and stabilize the self-assembled structure (Figure 1B). $(FFiX)_2$ peptides were synthesized by a liquid-phase method, and their self-assembling properties including hydrogelation were investigated. In addition, peptide hydrogels were applied to cell culture materials.

2 | EXPERIMENTAL SECTION

2.1 | General

All chemicals and solvents were of reagent or HPLC grade and were used without further purification. Amino acid derivatives were purchased from Watanabe Chemical Industries, Ltd (Japan). Other chemical reagents and solvents were purchased from Fujifilm Wako Pure Chemical (Japan) and Tokyo Chemical Industry (Japan). NMR measurement was conducted using Agilent UNITY-INOVA-400. MALDI-TOF MS measurement was conducted using MALDI-8020 (Shimadzu).

2.2 | Synthesis of self-assembling peptides

2.2.1 | Compound 1

Compound **1** was synthesized according to the previous report with modification.[19] To a suspended solution of H-Phe-OBzl hydrochloride salt (19.26 g, 66 mmol) in 100 mL dry dichloromethane was added *N*,*N*-diisopropylethylamine (DIPEA) (11.5 mL, 66 mmol) and carbonyldiimidazole (4.86 g, 30 mmol) at ice-water bath. After stirring at room temperature for 18 hours, the reaction mixture was concentrated under reduced pressure. The residue was diluted with 450 mL ethyl acetate/tetrahydrofuran (2/1) and the organic phase was washed with 1 M HCl_{aq} (100 mL × 2), sat $NaHCO_{3aq}$ (100 mL × 1) and sat $NaCl_{aq}$ (100 mL × 1). The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. After drying in vacuo, compound **1** was obtained as a white solid (14.15 g, 26.4 mmol, yield 88%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.32 (6H, m), 7.30-7.25 (4H, m), 7.20-7.14 (6H, m), 6.99-6.96 (4H, m), 5.15-5.04 (4H, m), 4.97 (2H, d, *J* = 7.6 Hz), 4.83-4.78 (2H, m), 3.07-2.99 (4H, m).

2.2.2 | Compound 2

Compound **2** was synthesized according to the previous report with modification.[19] Compound **1** (10.73 g, 20 mmol) was dissolved in 150 mL methanol and 150 mL ethyl acetate with heating at 50 °C. After cooling to room temperature, to this solution was added catalytic amount of 10% palladium on carbon, and the reaction mixture was stirred at room temperature under H_2 atmosphere. After stirring for 6 hours, the reaction mixture was filtrated on Celite, and the filtrate was concentrated under reduced pressure. The residue was solidified with excess diethyl ether to give compound **2** as a white solid (7.06 g, 19.8 mmol, yield 99%).

^{1}H NMR (400 MHz, DMSO-d_6): δ 6.87-6.77 (10H, m), 4.11 (2H, t, *J* = 5.6 Hz), 2.74-2.69 (2H, m), 2.59-2.54 (2H, m).

2.2.3 | Compound 3

Compound **3** was synthesized according to the previous report with modification.[20] To a solution of Boc-Orn(Z)-OH (9.16 g, 25 mmol) in 75 mL dry dichloromethane was added 4-dimethylaminopyridine (153 mg, 1.25 mmol), *tert*-butanol (50 mL, 527 mmol) and Boc_2O (8.6 mL, 37.5 mmol) at ice-water bath. After stirring at ice-water bath for 30 minutes and at room temperature for 4.0 hours, the reaction mixture was concentrated under reduced pressure. The residue was

purified by silica gel column chromatography (hexane/ethyl acetate = 7/3) to give compound **3a** as a colorless clear oil (7.24 g, 17.1 mmol, yield 68%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.29 (5H, m), 5.09 (2H, s), 5.06 (1H, bs), 4.88 (1H, bs), 4.18-4.15 (1H, m), 3.22 (2H, q, *J* = 6.4 Hz), 1.85-1.78 (1H, m), 1.67-1.49 (3H, m), 1.46 (9H, s), 1.44 (9H, s).

To a solution of Boc-Lys(Z)-OH (11.4 g, 30 mmol) in 90 mL dry dichloromethane was added 4-dimethylaminopyridine (184 mg, 1.5 mmol), *tert*-butanol (60 mL, 632 mmol) and Boc_2O (10.3 mL, 45 mmol) at ice-water bath. After stirring at ice-water bath for 30 minutes and at room temperature for 4.0 hours, the reaction mixture was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (hexane/ethyl acetate = 7/3) to give compound 3b as a colorless clear oil (9.17 g, 21 mmol, yield 70%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36-7.29 (5H, m), 5.09 (2H, s), 5.05 (1H, d, *J* = 6.4 Hz), 4.81 (1H, bs), 4.18-4.13 (1H, m), 3.19 (2H, q, *J* = 6.4 Hz), 1.83-1.72 (1H, m), 1.65-1.49 (3H, m), 1.46 (9H, s), 1.43 (9H, s), 1.39-1.25 (2H, m).

2.2.4 | Compound 4

Compound **4** was synthesized according to the previous report with modification.[21] To a solution of compound **3a** (5.5 g, 13 mmol) in 30 mL MeOH was added catalytic amount of 10% palladium on carbon, and the mixture was stirred at room temperature under H_2 atmosphere. After stirring for 7.0 hours, the reaction mixture was filtrated on Celite, and the filtrate was concentrated under reduced pressure. After dry in vacuo, compound **4a** was obtained as a white amorphous solid (3.4 g, 11.8 mmol, yield 91%). Compound **4a** was directly used in the next step.

To a solution of compound **3b** (6.3 g, 14.4 mmol) in 30 mL MeOH was added catalytic amount of 10% palladium on carbon, and the mixture was stirred at room temperature under H_2 atmosphere. After stirring for 7.0 hours, the reaction mixture was filtrated on Celite, and the filtrate was concentrated under reduced pressure. After dry in vacuo, compound **4b** was obtained as a white amorphous solid (4.24 g, 14 mmol, yield 97%). Compound **4b** was directly used in the next step.

2.2.5 | Compound 5

Compound **5** was synthesized according to the previous report with modification.[21] To a solution of compound **4a** (3.4 g, 11.8 mmol), Z-Phe-OH (3.21 g, 10.7 mmol), and HOBt H_2O (1.81 mg, 11.8 mmol) in 10 mL dry dichloromethane was added DIPEA (2.05 mL, 11.8 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide Hydrochloride (EDCI HCl) (2.26 g, 11.8 mmol) at ice-water bath. After stirring at ice-water bath for 1.0 hour and room temperature for 10 hours, the reaction mixture was concentrated under reduced pressure. The residue was diluted with 300 mL ethyl acetate and the organic phase was washed with 10% aqueous citric acid (150 mL × 2), sat $NaHCO_{3aq}$ (150 mL × 2) and sat $NaCl_{aq}$ (150 mL × 1). The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. After drying in vacuo, compound **5a** was obtained as a white amorphous solid (6.38 g, 11.2 mmol, yield 95%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.18 (10H, m), 5.96 (1H, bs), 5.40 (1H, bs), 5.12-5.05 (3H, m), 4.38-4.34 (1H, m), 4.18-4.09 (1H, m), 3.27-3.10 (3H, m), 3.03-2.98 (1H, m), 1.73-1.55 (2H, m), 1.59-1.51 (1H, m), 1.50-1.47 (2H, m), 1.46 (9H, s), 1.44 (9H, s).

To a solution of compound **4b** (4.24 g, 14 mmol), Z-Phe-OH (3.72 g, 12.4 mmol), and HOBt H_2O (2.15 g, 14 mmol) in 15 mL dry dichloromethane was added DIPEA (2.44 mL, 14 mmol) and EDCI HCl (2.67 g, 14 mmol) at ice-water bath. After stirring at ice-water bath for 1.0 hour and room temperature for 10 hours, the reaction mixture was concentrated under reduced pressure. The residue was diluted with 300 mL ethyl acetate and the organic phase was washed with 10% aqueous citric acid (150 mL × 2), sat $NaHCO_{3aq}$ (150 mL × 2) and sat $NaCl_{aq}$ (150 mL × 1). The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. After drying in vacuo, compound **5b** was obtained as a white solid (8.11 g, 13.9 mmol, yield 99%).

^{1}H NMR (400 MHz, $CDCl_3$): 7.38-7.18 (10H, m), 5.68 (1H, bs), 5.40 (1H, bs), 5.09 (2H, s), 5.06 (1H, bs), 4.35-4.30 (1H, m), 4.13-4.06 (1H, m), 3.14-3.10 (3H, m), 3.03-2.98 (1H, m), 1.74-1.66 (1H, m), 1.59-1.50 (1H, m), 1.46 (9H, s), 1.44 (9H, s), 1.38-1.30 (2H, m), 1.28-1.17 (2H, m).

2.2.6 | Compound 6

To a solution of compound **5a** (1.70 g, 3.0 mmol) in 15 mL methanol was added catalytic amount of 10% palladium on carbon, and the mixture was stirred at room temperature under N_2 atmosphere. After stirring for 5.0 hours, the reaction mixture was filtrated on Celite, and the filtrate was concentrated under reduced pressure. After drying in vacuo, compound **6a** was obtained as a white amorphous solid (1.28 g, 2.95 mmol, yield 98%). Compound **6a** was directly used in the next step.

To a solution of compound **5b** (1.75 g, 3.0 mmol) in 15 mL methanol was added catalytic amount of 10% palladium on carbon, and the mixture was stirred at room temperature under N_2 atmosphere. After stirring for 5.0 hours, the reaction mixture was filtrated on Celite, and the filtrate was concentrated under reduced pressure. After drying in vacuo, compound **6b** was obtained as a white amorphous solid (1.34 g, 2.99 mmol, yield 99%). Compound **6b** was directly used in the next step.

2.2.7 | Compound 7

To a solution of compound **6a** (1.28 g, 2.95 mmol) in 5 mL dry DMF was added compound **2** (476 mg, 1.34 mmol) and OxymaPure (417 mg, 2.95 mmol) at room temperature. To this mixture was added

DIPEA (511 μL, 2.95 mmol) and EDCI HCl (563 mg, 2.95 mmol) at ice-water bath. After stirring at ice-water bath for 1.0 hour and at room temperature for 23 hours, the reaction mixture was concentrated under reduced pressure. The residue was diluted with 400 mL ethyl acetate/tetrahydrofuran (4/1) and the organic phase was washed with 10% aqueous citric acid (120 mL × 2), sat $NaHCO_{3aq}$ (120 mL × 2) and sat $NaCl_{aq}$ (100 mL × 1). The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (chloroform/methanol = from 30/1 to 25/1 [v/v]). After drying in vacuo, compound **7a** was obtained as a white solid (1.03 g, 0.87 mmol, yield 65%).

^{1}H NMR (400 MHz, DMSO-d_6): 8.05 (2H, d, *J* = 8.4 Hz), 7.82 (2H, t, *J* = 5.6 Hz), 7.23-7.06 (22H, m), 6.31 (2H, d, *J* = 7.2 Hz), 4.45-4.39 (2H, m), 4.31-4.26 (2H, m), 3.76-3.71 (2H, m), 3.07-3.00 (2H, m), 2.98-2.77 (8H, m), 2.70-2.65 (2H, m), 1.59-1.39 (8H, m), 1.38 (36H, bs).

MALDI-TOF MS: *m/z obs*. 1213.90, *calcd*. 1213.65 $[M + Na]^+$.

To a solution of compound **6b** (1.34 g, 2.99 mmol) in 5 mL dry DMF was added compound **2** (485 mg, 1.36 mmol) and OxymaPure (424 mg, 2.99 mmol) at room temperature. To this mixture was added DIPEA (520 μL, 2.99 mmol) and EDCI HCl (572 mg, 2.99 mmol) at ice-water bath. After stirring at ice-water bath for 1.0 hour and at room temperature for 23 hours, the reaction mixture was concentrated under reduced pressure. The residue was diluted with 400 mL ethyl acetate/tetrahydrofuran (4/1) and the organic phase was washed with 10% aqueous citric acid (120 mL × 2), sat $NaHCO_{3aq}$ (120 mL × 2) and sat $NaCl_{aq}$ (100 mL × 1). The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (chloroform/methanol = from 30/1 to 25/1 [v/v]). After drying in vacuo, compound **7b** was obtained as a white solid (1.14 g, 0.94 mmol, yield 69%).

^{1}H NMR (400 MHz, DMSO-d_6): 8.05 (2H, d, *J* = 8.4 Hz), 7.80 (2H, t, *J* = 5.6 Hz), 7.24-7.10 (20H, m), 7.05 (2H, d, *J* = 8.0 Hz), 6.33 (2H, d, *J* = 7.2 Hz), 4.44-4.38 (2H, m), 4.31-4.26 (2H, m), 3.73-3.68 (2H,m), 3.09-3.01 (2H, m), 2.94-2.78 (8H, m), 2.71-2.66 (2H, m), 1.59-1.47 (4H, m), 1.38 (18H, s), 1.37 (18H, s), 1.29-1.11 (8H, m).

MALDI-TOF MS: *m/z obs*. 1241.72, *calcd*. 1241.68 $[M + Na]^+$.

2.2.8 | Compound 8

Compound **7a** (1.19 g, 1.0 mmol) was dissolved in 10 mL trifluoroacetic acid and the mixture was stirred at room temperature. After stirring for 1.5 hours, the mixture was concentrated under reduced pressure. To the residue was added 50 mL diethyl ether, and the precipitate was collected by centrifugation (3000 rpm, for 10 minutes). The solid was suspended in 50 mL diethyl ether with sonication and collected by centrifugation (3000 rpm, for 10 minutes). After dry in vacuo, compound **8a** was obtained as a white solid (1.02 g, 0.92 mmol, yield 92%).

^{1}H NMR (400 MHz, DMSO-d_6): 8.24-7.86 (8H, m), 7.24-7.10 (20H, m), 6.40 (2H, d, *J* = 7.2 Hz), 4.42-4.36 (2H, m), 4.26-4.21 (2H, m), 3.81-3.78 (2H, m), 3.11-2.89 (6H, m), 2.84-2.78 (4H, m), 2.67-2.62 (2H, m), 1.77-1.66 (4H, m), 1.57-1.39 (4H, m).

MALDI-TOF MS: *m/z obs*. 879.21 and 901.22, *calcd*. 879.44 $[M + H]^+$ and 901.42 $[M + Na]^+$.

Compound **7b** (1.22 g, 1.0 mmol) was dissolved in 10 mL trifluoroacetic acid and the mixture was stirred at room temperature. After stirring for 1.5 hours, the mixture was concentrated under reduced pressure. To the residue was added 50 mL diethyl ether, and the precipitate was collected by centrifugation (3000 rpm, for 10 minutes). The solid was suspended in 50 mL diethyl ether with sonication and collected by centrifugation (3000 rpm, for 10 minutes). After dry in vacuo, compound **8b** was obtained as a white solid (1.08 g, 0.95 mmol, yield 95%).

^{1}H NMR (400 MHz, DMSO-d_6): 8.19-7.76 (8H, m), 7.23-7.09 (20H, m), 6.48 (2H, d, *J* = 7.2 Hz), 4.42-4.33 (2H, m), 4.22-4.18 (2H, m), 3.61 (2H, bs), 3.01-2.94 (6H, m), 2.98-2.80 (4H, m), 2.71-2.65 (2H, m), 1.71 (4H, bs), 1.32 (8H, bs).

MALDI-TOF MS: *m/z obs*. 907.18 and 929.18, *calcd*. 907.47 $[M + H]^+$ and 929.45 $[M + Na]^+$.

2.3 | Circular dichroism spectroscopy

$(FFiX)_2$ peptides (TFA salt) were dissolved in 100 μL of an aqueous 40 mM NaOH solution at a concentration of 10 mM. The peptide solution (100 μL) was mixed with 100 μL of 20 mM aqueous HCl solution for neutralization, and the neutralized peptide solution (5.0 mM) was quickly diluted with ultrapure water and 20 mM Tris-HCl buffer (pH 7.2) to prepare peptide solutions at 100 μM, 200 μM, 300 μM, 500 μM and 1000 μM in 10 mM Tris-HCl buffer (pH 7.2). The diluted solutions were incubated at 25 °C for 24 hours. Circular dichroism (CD) spectra were recorded on a JASCO J-1100 spectropolarimeter using a quartz cell with a 0.1 cm path length at 25 °C.

Neutralized peptide solution (5.0 mM) was prepared as mentioned above and quickly diluted with ultrapure water and 20 mM Tris-HCl solution (pH 4.2, 7.2 or 10.2) to prepare 300 μM peptide solution in 10 mM Tris-HCl (pH 4.2, 7.2 or 10.2). The diluted solutions were incubated at 25 °C for 24 hours.

2.4 | Transmission electron microscopy

A $(FFiO)_2$ solution at a concentration of 300 μM was prepared in 10 mM Tris-HCl buffer (pH 7.2). $(FFiK)_2$ solutions at a concentration of 300 μM were prepared in 10 mM Tris-HCl solutions (pH 4.2, 7.2 and 10.2). These solutions were incubated at 25 °C for 24 hours. A collodion-coated copper EM grid was glow discharged for 40 seconds at 3 mA using an IB2 ion coater. A 10 μL drop of the peptide solution was applied to the freshly glow discharged grid for 1 minute to adsorb peptide nanofibers. The excess solution was removed by capillary action

with filter paper, and the grid was then washed twice with 10 μL of ultrapure water. After removal of the ultrapure water, the grid was stained for 2 minutes with EM Stainer (Nisshin EM) that was diluted 4-fold. The stained grid was blotted with filter paper and allowed to dry at room temperature. Transmission electron microscopy (TEM) images were taken with a JEM 1400Plus (JEOL) instrument operated at 80 kV.

2.5 | Hydrogelation test

$(FFiO)_2$ (20 mg, 18.0 μmol) or $(FFiK)_2$ (20 mg, 17.6 μmol) was dissolved in 1.0 mL of a NaOH solution (4 equiv.) to prepare a 2.0 wt% peptide solution. The peptide solution (1.0 mL) was neutralized with 1.0 mL of an HCl solution (2 equiv.) to prepare a 1.0 wt% peptide solution. The neutralized peptide solution (1.0 wt%, 0.5 mL) was quickly mixed with 0.5 mL of 20 mM Tris-HCl buffer (pH 7.2) to prepare a 0.5 wt% peptide solution in 10 mM Tris-HCl buffer (pH 7.2). The 0.5 wt% peptide solution was quickly diluted with 10 mM Tris-HCl buffer (pH 7.2) to prepare peptide solutions at concentrations of 0.2 wt%, 0.1 wt% and 0.05 wt%. All peptide solutions were incubated at 25 °C for 24 hours with a stir bar.

$(FFiO)_2$ (8 mg, 7.2 μmol) or $(FFiK)_2$ (8 mg, 7.04 μmol) was dissolved in 1.0 mL of a NaOH solution (4 equiv.) to prepare a 0.8 wt% peptide solution. The peptide solution (1.0 mL) was neutralized with 1.0 mL of an HCl solution (2 equiv.) to prepare 0.4 wt% peptide solution. The neutralized peptide solution (0.4 wt%, 100 μL) was quickly mixed with 100 μL of sodium citrate/phosphate/borate buffer (each 20 mM, pH = 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 10.2, 11.2) or 100 μL of Dulbecco's modified Eagle medium (DMEM) containing phenol red to prepare peptide solutions at concentration of 0.2 wt%. All peptide solutions were incubated at 25 °C for 24 hours.

$(FFiO)_2$ (10 mg, 9.0 μmol) or $(FFiK)_2$ (10 mg, 8.8 μmol) was dissolved in 0.5 mL of a NaOH solution (4 equiv.) to prepare a 2.0 wt% peptide solution. The peptide solution (0.5 mL) was transferred to a tip-cut plastic syringe (5 mL) and neutralized with 0.5 mL of 20 mM Tris HCl buffer containing HCl (2 equiv.) to prepare a 1.0 wt% peptide solution. After 5 minutes of incubation, hydrogels were transferred onto a ruler for photography.

Nile red (NR) and thioflavin T (ThT) were dissolved in dimethylsulfoxide (DMSO) to prepare a 1.0 mM stock solution. $(FFiK)_2$ (5.0 mg, 4.4 μmol) was dissolved in 0.25 mL of a NaOH solution (4 equiv.) to prepare a 2.0 wt% peptide solution. Stock solutions of NR or ThT (1.0 μL) were added to the peptide solution (100 μL), respectively. The fluorophore-loaded peptide solution (2.0 wt%, 40 μL) was injected into 1.5 mL of phosphate-buffered saline (PBS, pH 7.2) in a quartz cell using a microsyringe (inner needle diameter of 0.15 mm) to fabricate hydrogel fibers. Hydrogel fibers were visualized by irradiation with UV light (354 nm).

2.6 | Rheology

Measurements were conducted on a TA Instruments Discovery Hybrid Rheometer HR 30 operating in oscillatory mode, with a 20 mm parallel plate geometry. Hydrogels (1.0 wt%) of $(FFiO)_2$ and $(FFiK)_2$ were prepared in a tip-cut plastic syringe (20 mL) with 24 hours incubation time. Then, hydrogel samples were transferred to the rheometer stage (20 mm) by slowly extruding. A dynamic frequency sweep was immediately performed over a range of frequencies from 0.1 to 100 rad s^{-1} at 0.1% constant strain at 25 °C.

2.7 | Cell culture

HepG2 cells were obtained from the RIKEN BRC. The cell line was tested and authenticated by the RIKEN BRC. HepG2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were cultured on Petri dishes and grown until reaching 80% confluence at 37 °C under 5% CO_2 condition.

2.8 | Cell culture on $(FFiK)_2$ hydrogels

An $(FFiK)_2$ peptide solution (2.0 wt%) was prepared as described above. The $(FFiK)_2$ peptide solution (50 μL) was added to a 96-well culture plate (EZ-BindShut-II, IWAKI), and mixed with DMEM (50 μL). Then, the peptide solution was incubated for 15 minutes to prepare a 1.0 wt% peptide hydrogel. DMEM (200 μL) was added on the peptide hydrogels and incubated for 15 minutes. After DMEM was removed, 1×10^3 HepG2 cells in DMEM (100 μL) supplemented with FBS and antibiotics were added on the hydrogels and cultured at 37 °C under 5% CO_2 for 5 days with the medium change every 2 days. HepG2 cells (1×10^3 cells) were cultured on tissue culture-treated plate (TCTP) as a control. Live cells and dead cells were stained with calcein-AM (2.0 μM) and propidium iodide (PI, 4.0 μM), respectively, after 1, 3 and 5 days cultivation according to manufacturer's instructions. The cells were observed with a fluorescence microscope (Ti-U-PH-1, Nikon). The fluorescence intensity of live cells stained with calcein was measured with a microplate fluorometer (ARVO MX, PerkinElmer). Measurement was conducted for three different wells. Statistical significance was indicated by $P < .05$.

2.9 | Cell culture on $(FFiK)_2$ hydrogel fibers

An $(FFiK)_2$ peptide solution (2.0 wt%) containing NR (10 μM) was prepared as described above, and 40 μL of the peptide solution was injected into 1.0 mL of DMEM in a 24-well culture plate (EZ-BindShut-II, IWAKI) using a microsyringe (inner needle diameter of 0.15 mm) to fabricate hydrogel fibers. The hydrogel fibers were incubated at 37 °C for 5 minutes. After the DMEM was carefully removed, 2.5×10^3 HepG2 cells in DMEM (100 μL) supplemented with FBS and antibiotics were added to the well and cultured at 37 °C under 5% CO_2 for 2 days. Live cells were stained with calcein-AM (2.0 μM) and observed with a fluorescence microscope.

3 | RESULTS AND DISCUSSION

3.1 | Molecular design and synthesis of short self-assembling peptides

$(FFiX)_2$ peptides were designed as short self-assembling peptides composed of six amino acids (Figure 1A). A diphenylalanine (FF) unit was used as a representative self-assembling motif,[14] and two FF units were connected via a urea bond that can form strong hydrogen bonds. A urea unit has been used for precise control of self-assembly of macrocycle,[22] tunable organogel/hydrogel formation,[23,24] and functionalized biomaterials.[25] However, there is very few applications of the urea unit to the design of self-assembling peptide composed of natural amino acids. Two ornithine residues (n = 0, $(FFiO)_2$) or lysine residues (n = 1, $(FFiK)_2$) were introduced at both ends via an isopeptide bond to display zwitterion units, because an amino acid framework can form intermolecular ion pairs.[26,27] Under physiological aqueous conditions, $(FFiX)_2$ peptides are expected to spontaneously assemble by a hydrophobic interaction between FF units, a hydrogen bonding network formed due to urea bonds and electrostatic interactions at both ends (Figure 1B). In addition, the $(FFiX)_2$ peptides were designed to have a symmetrical structure for their easy synthesis in the liquid phase. Ac-FFiK, which does not have a urea unit, was designed and used for comparison (Figure 1A). These peptides were synthesized by liquid phase method according to the scheme shown in Figure 2 and Figure S1. We have established a synthetic method of $(FFiX)_2$ peptides on a scale larger than that of the representative solid-phase method. In the case of $(FFiK)_2$, the peptide could be synthesized on a scale of 20 mmol (approximately 22 g) scale with high purity. This is one of the advantages of short symmetric self-assembling peptides that can be synthesized in the liquid phase.

3.2 | Secondary structure analysis by CD spectroscopy

CD spectra were measured to evaluate the secondary structure of $(FFiX)_2$ peptides in 10 mM Tris-HCl buffer (pH 7.2) (Figure 3). $(FFiO)_2$ at 100 and 200 μM showed a positive maximum around 220 nm and a negative maximum around 205 nm, indicating a random coil structure, which is characteristic of phenylalanine-based self-assembling peptides (Figure 3A).[28] However, 300 μM $(FFiO)_2$ showed a negative maximum around 215 nm with a shoulder peak at 205 nm. This result suggests that $(FFiO)_2$ assembled into a β-sheet structure. The peak at

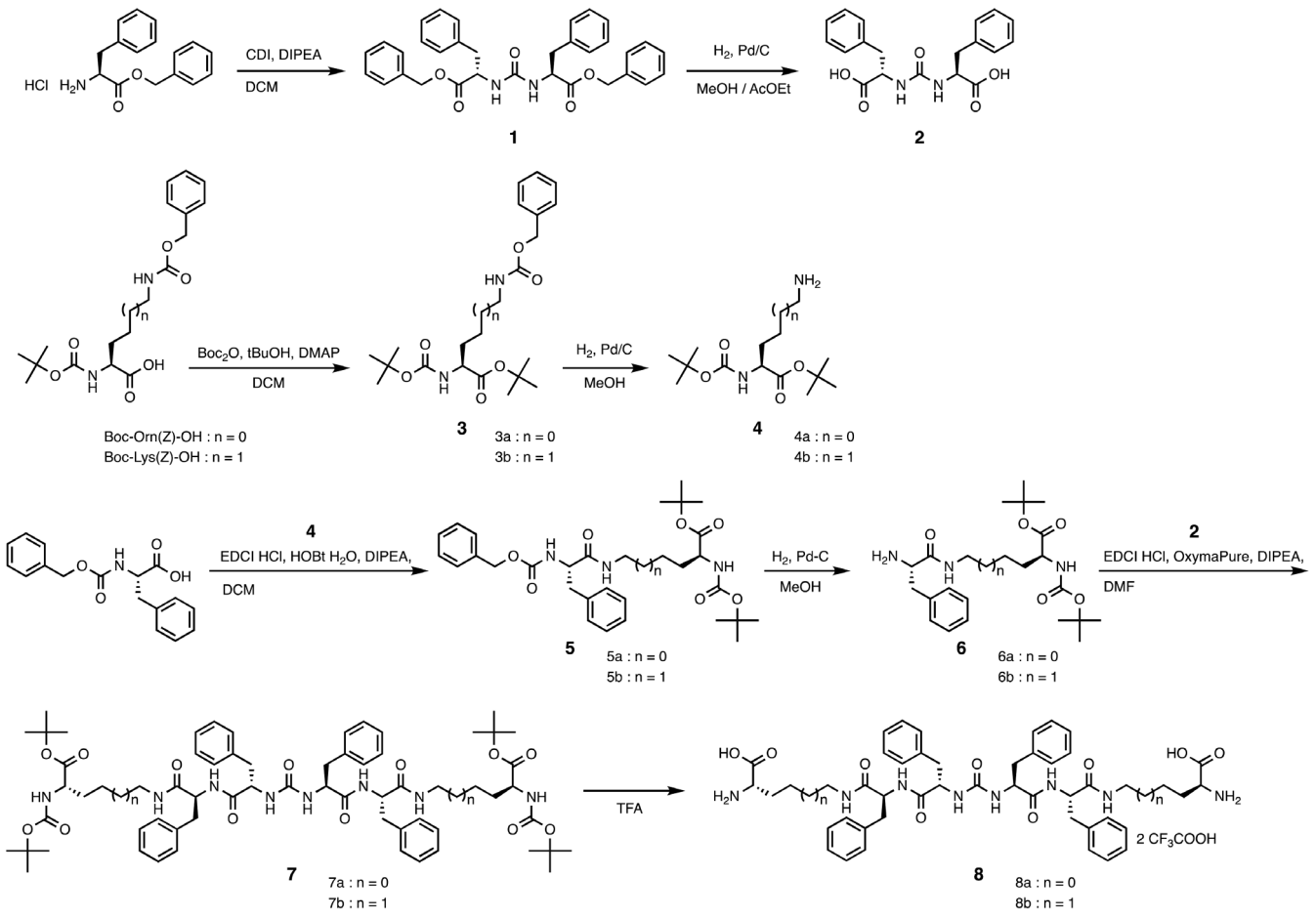


FIGURE 2 Synthesis scheme of $(FFiO)_2$ and $(FFiK)_2$

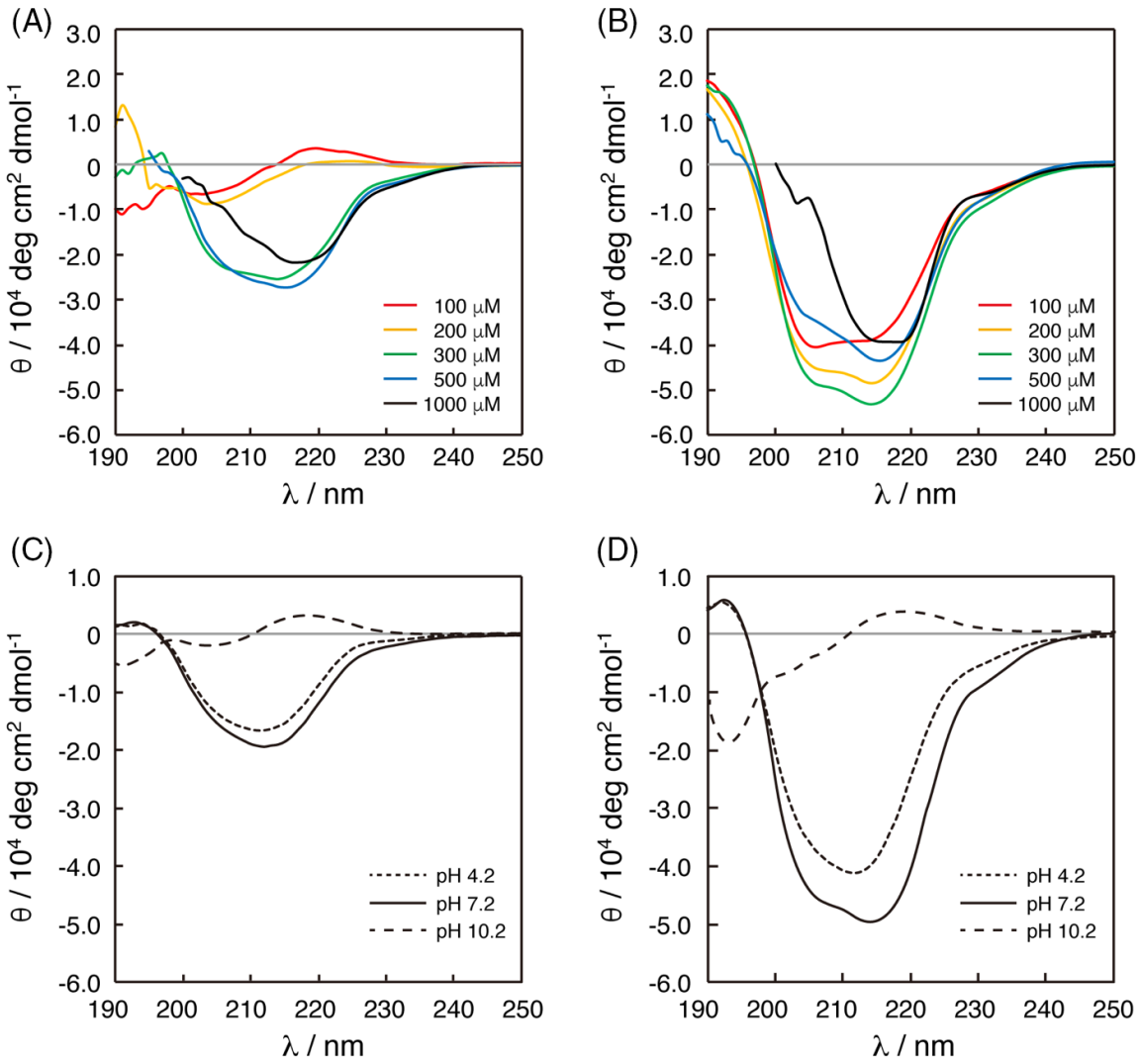


FIGURE 3 Circular dichroism spectra of, A, $(FFiO)_2$ and B, $(FFiK)_2$. The concentration of peptides was varied from 100 μM to 1000 μM at 25 °C in 10 mM Tris-HCl buffer (pH 7.2). Circular dichroism spectra of, C, $(FFiO)_2$ and D, $(FFiK)_2$ at different pH condition. The concentration of peptides was 300 μM at 25 °C in 10 mM Tris-HCl solution (pH 4.2, 7.2 and 10.2)

215 nm is derived from the hydrogen bonding of amide bonds in β-sheet structure, and the peak at 205 nm is derived from a π-π interaction between phenyl groups of side chains or distorted β-sheet structure.[9,28] When the peptide concentration increased, the negative peak shifted from 214 nm to 216 nm (500 μM) and 218 nm (1000 μM) with the peak intensity increasing. In addition, the shoulder peak around 205 nm almost disappeared at 1000 μM $(FFiO)_2$. These results indicate that $(FFiO)_2$ at high concentrations formed many hydrogen bonds and that a hydrogen bonding network contributed to the formation of a stable β-sheet structure.

In contrast, $(FFiK)_2$ at 100 μM showed negative maxima around 215 nm and 205 nm (Figure 3B). This result suggests that $(FFiK)_2$ could assemble into a β-sheet structure at a lower concentration than $(FFiO)_2$. When the peptide concentration increased, the CD signal intensity decreased up to a $(FFiK)_2$ concentration of 300 μM, and the signal intensity at 215 nm became larger than that at 205 nm. This result indicates that $(FFiK)_2$ also formed many hydrogen bonds with increasing peptide concentration. In addition, at a concentration of 300 μM, $(FFiK)_2$ exhibited a larger CD signal intensity than $(FFiO)_2$, indicating that $(FFiK)_2$ formed a more stable β-sheet structure than $(FFiO)_2$. Although $(FFiO)_2$ and $(FFiK)_2$ differ by just one methylene group, a subtle structural difference might affect the electrostatic interaction network at the edge of both peptides. At higher concentrations of $(FFiK)_2$, the negative peak shifted from 215 nm to 217 nm (500 μM) and 219 nm (1000 μM), and the peak intensity increased with the disappearance of the shoulder peak around 205 nm as observed with $(FFiO)_2$. These results suggest that $(FFiK)_2$ also assembled into a stable β-sheet structure in a concentration dependent manner similar to $(FFiO)_2$. In addition, IR spectra also supported that both $(FFiO)_2$ and $(FFiK)_2$ could assemble into β-sheet structure, because they showed peaks around 1630 cm^{-1} characteristic with a β-sheet structure (Figure S2).[29]

Notably, 100 μM of $(FFiK)_2$ assembled into a β-sheet structure, whereas a similar designed peptide, Ac-$(KEFFFFKE)_2$-NH_2, which has

a hydrophobic domain and zwitterionic ends, adopted random coil structure at 200 μM.[30] Although it is difficult to directly compare the two peptides, the urea unit of $(FFiK)_2$ might contribute to its superior self-assembling ability.

CD spectra of $(FFiX)_2$ peptides were measured under different pH conditions (pH 4.2, 7.2 and 10.2) to evaluate the contribution of electrostatic interactions at the edge of peptides to self-assembly (Figure 3C,D). The concentration of both peptides was fixed at 300 μM. $(FFiO)_2$ and $(FFiK)_2$ showed a positive maximum around 220 nm and a negative maximum around 195 nm at pH 10.2, indicating that neither peptide self-assembled into β-sheet structures at pH 10.2. A similar CD spectrum was reported in the trigonal-$(FKFE)_2$ peptide at pH 11.[31] Since $(FFiO)_2$ and $(FFiK)_2$ can theoretically have a net charge of −2 at pH 10.2, electrostatic repulsion between the peptides disrupted the self-assembly. On the other hand, $(FFiO)_2$ and $(FFiK)_2$ showed a negative maximum around 210 nm at pH 4.2. This result implies that both peptides formed β-sheet-like aggregates under acidic condition. Although $(FFiO)_2$ and $(FFiK)_2$ have a net charge of +2 at pH 4.2, a hydrogen bond between —COOH and $—NH_3^+$ might contribute to promote self-assembly over electrostatic repulsion. Both $(FFiO)_2$ and $(FFiK)_2$ showed a negative maximum around 215 nm at pH 7.2. These results suggest that $(FFiX)_2$ peptides assemble into a β-sheet structure in response to pH change. The electrostatic interactions of intermolecular ion pairs at the edge of peptides contributes to stable β-sheet formation under physiological pH conditions.

3.3 | Nanostructure analysis by TEM observation

Self-assembled nanostructures of $(FFiX)_2$ peptides were observed by TEM. The concentration of both peptides in 10 mM Tris-HCl buffer (pH 7.2) was fixed at 300 μM. Both $(FFiO)_2$ and $(FFiK)_2$ assembled into nanofibers with an approximately 8 nm width and a more than 500 nm length at pH 7.2 (Figure 4A,B). Because the length of $(FFiX)_2$ peptides in an extended conformation is approximately 3.4 nm, this result indicates that β-sheets assembled from both peptides stacked by hydrophobic interactions between phenylalanine residues to form wider nanofibers. In particular, $(FFiK)_2$ exhibited densely bundled and networked nanofibers (Figure 4B). This result suggests that $(FFiK)_2$ can form more fibers than $(FFiO)_2$, which is consistent with the superior self-assembling property of $(FFiK)_2$ compared to $(FFiO)_2$ from the CD study.

The effect of pH on the self-assembly of $(FFiK)_2$ was investigated by TEM. The peptide concentration was fixed at 300 μM, and the peptide solutions were incubated at pH 4.2 (Figure 4C) or pH 10.2 (Figure 4D) in a 10 mM Tris solution. Under the acidic condition, $(FFiK)_2$

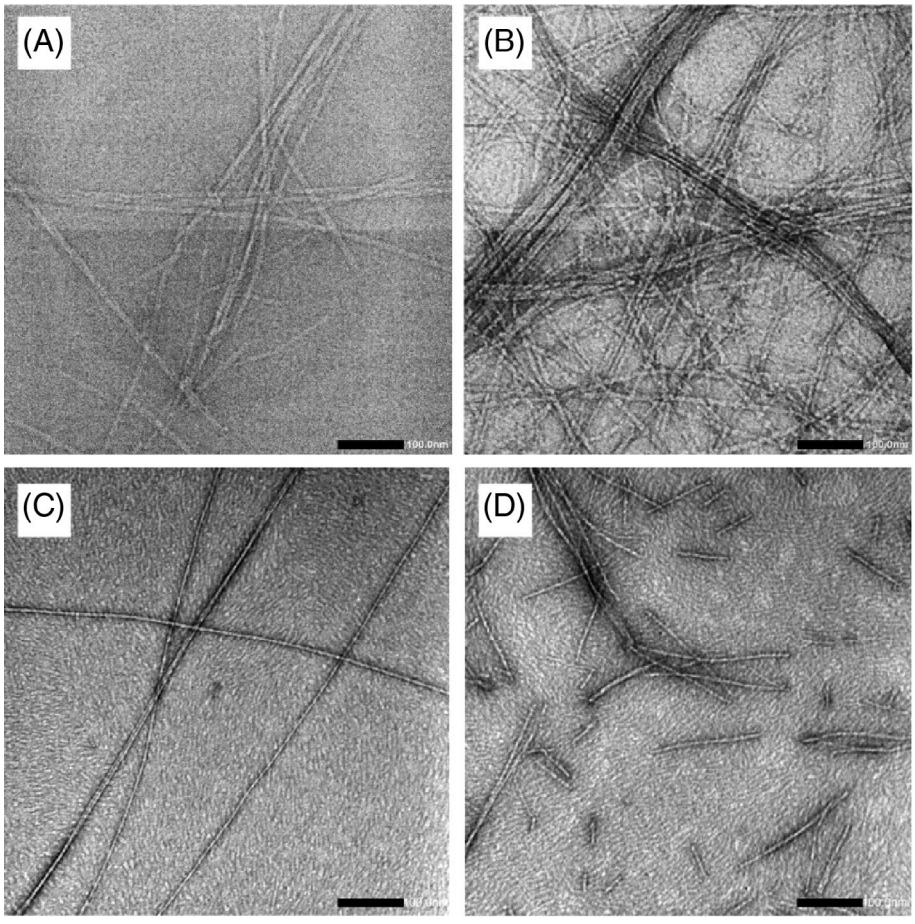


FIGURE 4 TEM images of, A, $(FFiO)_2$ at pH 7.2, B, $(FFiK)_2$ at pH 7.2, C, $(FFiK)_2$ at pH 4.2 and D, $(FFiK)_2$ at pH 10.2. The concentration of peptides was 300 μM. Scale bar is 100 nm

was present as long nanofibers with an approximately 8 nm width; however, no bundled nanofibers were observed. This result does not contradict the CD results, which indicated that $(FFiK)_2$ could form β-sheet-like aggregate at pH 4.2. However, detail assembly mode of $(FFiK)_2$ at pH 4.2 might be different to that at pH 7.2, because the peptide has a net charge of +2 at pH 4.2, and electrostatic repulsion between peptides could disrupt well-packed β-sheet assembly. Since $(FFiK)_2$ possibly would form micelle-like nanofibers that did not have well aligned surface at pH 4.2, nanofibers did not form bundled structures. In addition, nanofibers would have many positive charge at pH 4.2, and electrostatic repulsion between fibers might inhibit networked structures of nanofibers under acidic condition. On the other hand, $(FFiK)_2$ was present as short fibrils under basic condition. Since $(FFiK)_2$ showed a random coil structure at pH 10.2 in the CD experiment, $(FFiK)_2$ could not form a stable nanofiber structure. Based on the above results, $(FFiK)_2$ self-assembled into highly networked nanofibrous structures in response to pH.

3.4 | Hydrogelation test of self-assembling peptides

The hydrogelation properties of $(FFiX)_2$ peptides were investigated in 10 mM Tris-HCl buffer (pH 7.2). First, the peptide was dissolved in a NaOH solution (4 equiv. to peptide) at 2.0 wt%, because self-assembly is suppressed under basic condition. The peptide solution was neutralized with the proper amount of an HCl solution and quickly diluted with Tris-HCl buffer to prepare 0.5, 0.2, 0.1 and 0.05 wt% solutions. After 24 hours of incubation at 25 °C with a stir bar, hydrogelation was confirmed by an inverted method (Figure 5A). $(FFiO)_2$ at concentrations of 0.5, 0.2 and 0.1 wt% formed stable self-supporting hydrogels. Although the $(FFiO)_2$ hydrogel at 0.5 wt% was slightly opaque, the 0.2 and 0.1 wt% $(FFiO)_2$ hydrogel were transparent. At a concentration of 0.05 wt%, a partial gel was observed. In contrast, $(FFiK)_2$ at all concentration examined formed stable and transparent self-supporting hydrogels. These results and the results of CD studies and TEM observation suggest that both $(FFiO)_2$ and $(FFiK)_2$ self-assembled into networked nanofibers through a β-sheet structure and formed stable self-supporting hydrogels. In addition, $(FFiK)_2$ exhibited a lower critical hydrogelation concentration than $(FFiO)_2$ due to its superior self-assembling ability as demonstrated in the CD experiment. Hydrogelation of the Ac-FFiK peptide was also tested at concentrations of 0.2, 0.5, 1.0, 1.5 and 2.0 wt% (Figure S3). Ac-FFiK did not form hydrogels when at a concentration below 0.5 wt % and formed opaque hydrogels when at a concentration above 1.0 wt%. This result implies that the symmetric structure of $(FFiX)_2$ with a urea unit is important for transparent hydrogel formation at low concentrations.

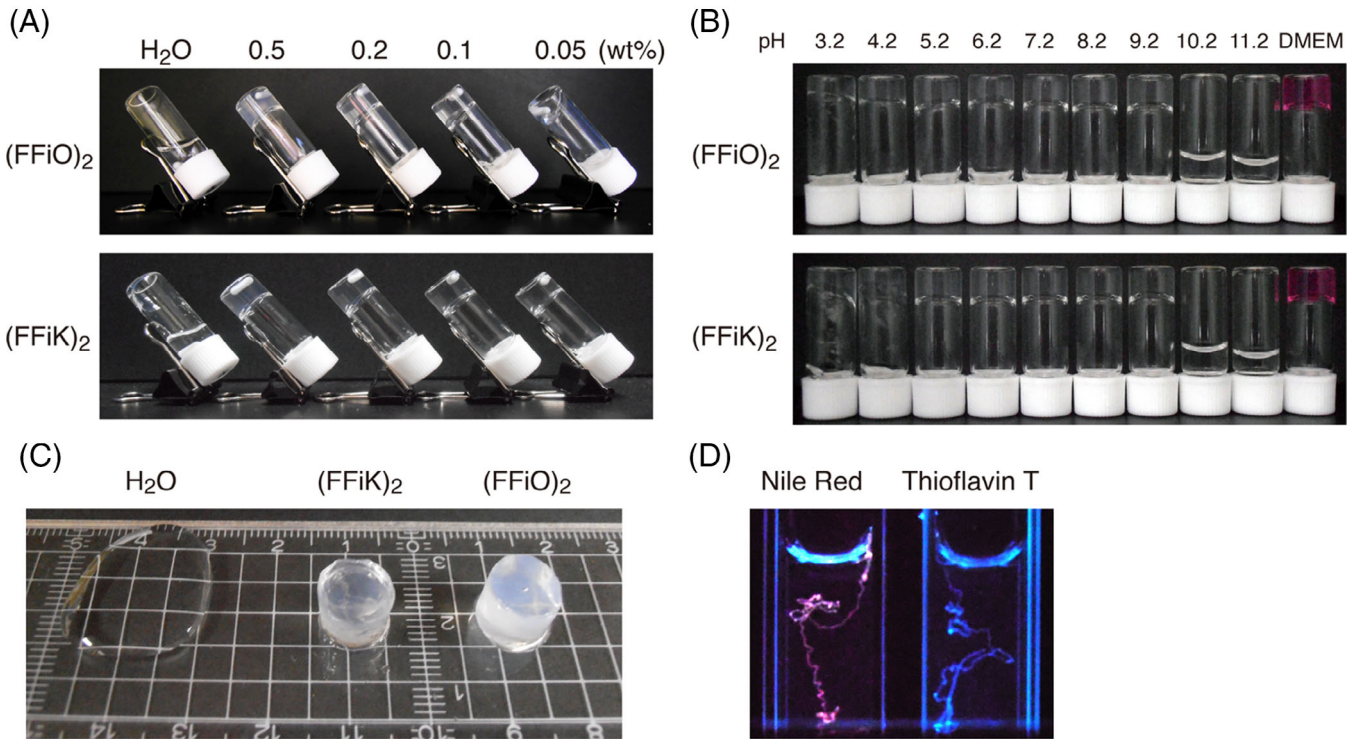


FIGURE 5 Photographs of $(FFiX)_2$ hydrogels. A, Concentration dependent hydrogelation of $(FFiX)_2$ peptides in 10 mM Tris-HCl buffer (pH 7.2) with stirring bar. The concentration of peptides was varied from 0.05 wt% to 0.5 wt%. B, pH dependent hydrogelation of $(FFiX)_2$ peptides in sodium citrate/phosphate/borate buffer (each 10 mM, pH 3.2-11.2). DMEM was also tested for hydrogelation of $(FFiX)_2$ peptides. The concentration of peptides was 0.2 wt%. C, Bulk hydrogels of $(FFiX)_2$ peptides. The concentration of peptides was 1.0 wt%. D, Hydrogel fibers of $(FFiK)_2$ fabricated by injection of 2.0 wt% $(FFiK)_2$ solution into PBS using microsyringe. Hydrogel fibers were stained with Nile Red (10 μM) or thioflavin T (10 μM) and visualized under UV light (354 nm) irradiation

Next, pH-responsive hydrogelation of $(FFiX)_2$ peptides was tested. Peptide solutions at a 0.2 wt% concentration were prepared using sodium citrate/phosphate/borate buffer (each 20 mM, pH 3.2-11.2) and incubated at 25 °C for 24 hours (Figure 5B). Both $(FFiO)_2$ and $(FFiK)_2$ formed stable and transparent hydrogels in the pH range from 5.2 to 9.2. Electrostatic interactions between peptides contribute to stabilizing self-assembly, because both ionic peptide termini maintain a zwitterionic form in this pH range. Under acidic conditions (pH 3.2 and 4.2), both peptides formed partial hydrogels, indicating that not enough of the peptide nanofiber network was formed to completely support the solvent. Although $(FFiK)_2$ could assemble into nanofibers at pH 4.2 as shown in Figure 4C, these nanofibers did not form bundled or networked structures. On the other hand, both $(FFiO)_2$ and $(FFiK)_2$ remained as a solution under basic conditions (pH 10.2 and 11.2). Because $(FFiK)_2$ could not form long nanofibers at pH 10.2 as shown in Figure 4D, neither peptide could form hydrogels. Based on these results, both $(FFiO)_2$ and $(FFiK)_2$ can form hydrogels in response to pH, and these peptide hydrogels are stable under physiological pH conditions. In addition, both $(FFiO)_2$ and $(FFiK)_2$ formed stable and transparent hydrogels when cell culture medium (DMEM) was used as a solvent (Figure 5B). This result indicates that $(FFiX)_2$ peptides can be used as cell culture materials.

Since solutions of $(FFiO)_2$ and $(FFiK)_2$ at a high concentration rapidly formed stable hydrogels in response to pH change, the hydrogels were cylindrical in shape in the plastic syringe. Basic peptide solutions (2.0 wt%) were neutralized by the proper amount of an HCl solution, and incubation for $(FFiK)_2$ for 1 minute and $(FFiO)_2$ for 5 minutes gave stable hydrogels (1.0 wt% for both peptides) (Figure 5C). These hydrogels maintained their structure for at least 1 month under high humidity. In addition, the $(FFiK)_2$ hydrogel was more transparent than the $(FFiO)_2$ hydrogel. Transparency of the $(FFiK)_2$ hydrogel was quite higher than that of the $(FFiO)_2$ hydrogel in the range from 400 nm to 700 nm (Figure S4). The detail reason for this difference was not determined; however, a subtle structural difference between $(FFiK)_2$ and $(FFiO)_2$ might affect the molecular packing in the self-assembled structures.

Rheological analysis was conducted to determine the storage modulus (G') and loss modulus (G'') for $(FFiO)_2$ and $(FFiK)_2$ hydrogels (1.0 wt%) (Figure 6). The $(FFiO)_2$ hydrogel showed higher G' (8925 ± 964 Pa) than G'' (1160 ± 358 Pa). The $(FFiK)_2$ hydrogel also showed higher G' (4489 ± 866 Pa) than G'' (842 ± 148 Pa). These results suggest that 1.0 wt% solutions of $(FFiO)_2$ and $(FFiK)_2$ meet the technical definition of a hydrogel.[32] Both $(FFiO)_2$ and $(FFiK)_2$ hydrogels exhibited large G' values more than 4000 Pa, while G'/G'' ratios of $(FFiO)_2$ and $(FFiK)_2$ hydrogels were 7.69 and 5.33, respectively. This result indicates that $(FFiO)_2$ and $(FFiK)_2$ hydrogels are soft gels to the extent possible for biological applications.

Because the hydrogelation of $(FFiK)_2$ was faster than that of $(FFiO)_2$, $(FFiK)_2$ was used to fabricate hydrogel fibers. Basic peptide solutions (2.0 wt%) containing fluorescent dyes were injected into PBS using a microsyringe (inner diameter = 0.15 mm) to prepare hydrogel fibers (Figure 5D). The hydrogel fibers were visualized by the

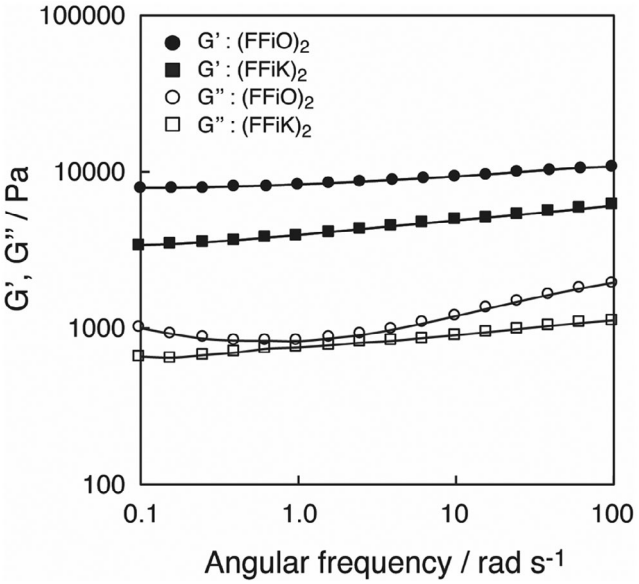


FIGURE 6 Rheological analysis of $(FFiO)_2$ and $(FFiK)_2$ hydrogels. Storage modulus (G') and loss modulus (G'') measured with frequency sweeps over 0.1-100 rad s^{-1}, 0.1% constant strain, at 25 °C

fluorescence of Nile red (NR) or thioflavin T under UV light irradiation, and the fiber width was estimated to be approximately 0.3 mm. This result indicates that the rapid self-assembly of $(FFiK)_2$ in response to pH change induced hydrogelation before diffusion of peptide. Nile red, an environmentally sensitive probe, was used to visualize the peptide hydrogel fibers, which showed strong fluorescence, suggesting that Nile red bound to the hydrophobic domain in the assembled $(FFiK)_2$ nanostructures. In addition, thioflavin T, a fluorescent probe used to detect assembled β-sheet structures, also visualized peptide hydrogel fibers with strong fluorescence, indicating that the hydrogel was composed of a β-sheet assembly of $(FFiK)_2$.

3.5 | Cell culture on $(FFiK)_2$ hydrogels

Since $(FFiK)_2$ exhibited hydrogelation properties superior to those of $(FFiO)_2$, $(FFiK)_2$ hydrogels were applied as a cell culture scaffold to confirm its cytocompatibility. The HepG2 cell line, a human hepatocyte carcinoma cell line, was used as a cellular model. $(FFiK)_2$ hydrogels (1.0 wt%) was prepared from DMEM in a 96-well plate and HepG2 cells (1×10^3 cells) were cultured on the hydrogel or tissue culture-treated plate (TCTP) for 5 days. After 1, 3 and 5 days of cultivation, live cells and dead cells were stained with calcein-AM (2.0 μM) and propidium iodide (PI, 4.0 μM), respectively. After 1 day of cultivation, HepG2 cells adhered to the surface of hydrogels (Figure S5a). Most HepG2 cells were stained with calcein, and a few cells were stained with PI (Figure S5b,c). These results indicate that the $(FFiK)_2$ hydrogel exhibits cell compatibility. HepG2 cells proliferated on the $(FFiK)_2$ hydrogel and formed spheroids after 3 days (Figure S5d,e) and 5 days (Figure 7A,B) of cultivation. During this culture period,

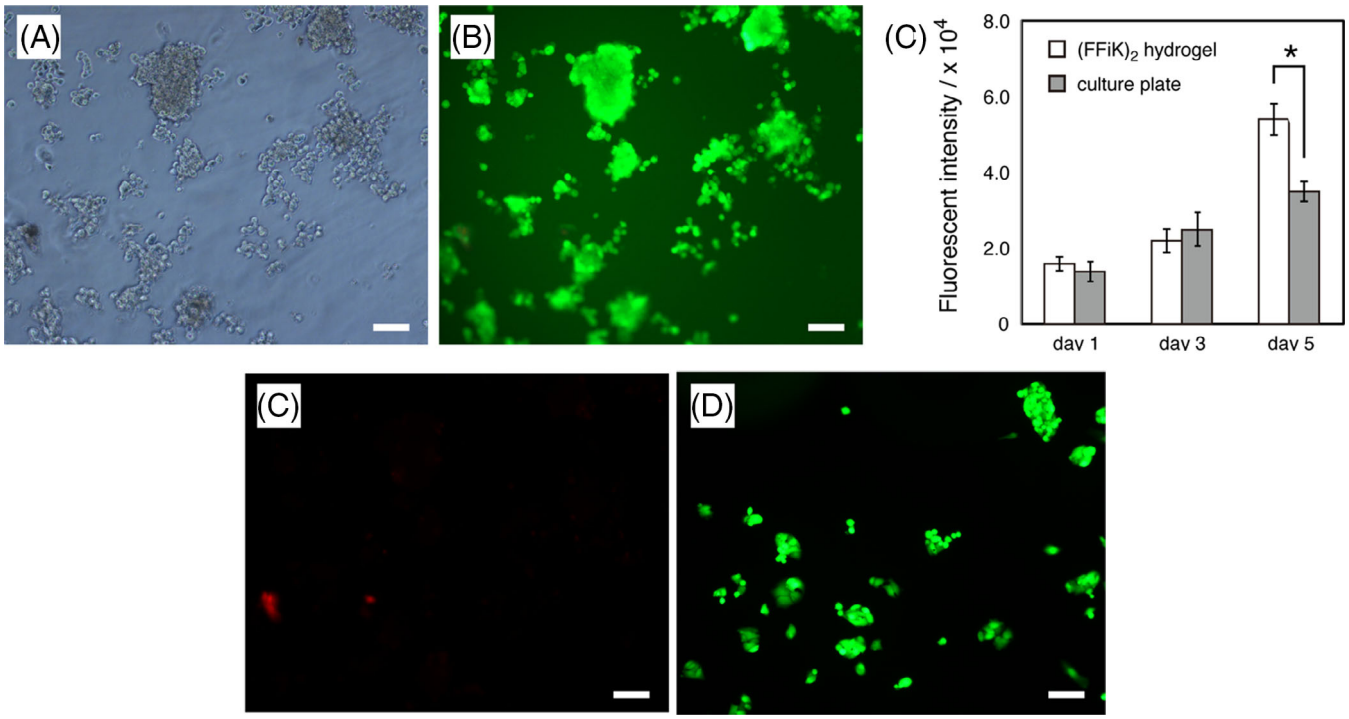
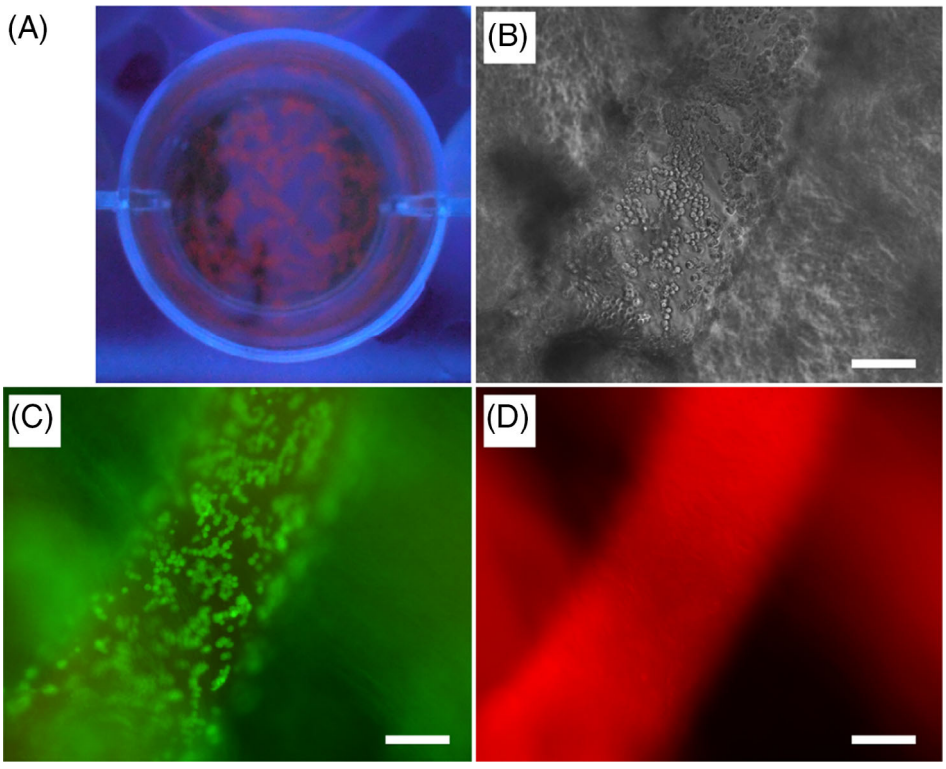


FIGURE 7 A, Bright filed image of HepG2 cells cultured on $(FFiK)_2$ hydrogel after 5 days. B and C, Fluorescence images of HepG2 cells cultured on $(FFiK)_2$ hydrogel after 5 days. Live cells were stained with Calcein (B) and dead cells were stained with PI (C). D, Fluorescence image of HepG2 cells on TCTP after 5 days. Live cells were stained with Calcein. Scale bar is 100 μm. E, Fluorescent intensity of Calcein-stained HepG2 cells cultured on $(FFiK)_2$ hydrogels and TCTP. $^*P < .05$

FIGURE 8 A, Photograph of $(FFiK)_2$ hydrogel fibers stained with NR (10 μM) in 24-well microplate. Hydrogel fibers were visualized under UV light (354 nm) irradiation. B, Bright filed image of HepG2 cells cultured on $(FFiK)_2$ hydrogel fibers after 2 days. C, Fluorescence image of HepG2 cells cultured on $(FFiK)_2$ hydrogel fibers after 2 days. D, Fluorescence image of $(FFiK)_2$ hydrogel fibers stained with NR (10 μM). Scale bar is 100 μm



significant cytotoxicity was not observed (Figure S5f and Figure 7C). In addition, HepG2 cells exhibited faster proliferation on the $(FFiK)_2$ hydrogel than on TCTP (Figure 7D). The fluorescence intensity from live cells on the $(FFiK)_2$ hydrogel was higher than that on TCTP. These results suggest that the $(FFiK)_2$ hydrogel could promote HepG2 cell proliferation.

Hydrogel fibers of $(FFiK)_2$ were also applied to culture of HepG2 cells. Hydrogel fibers stained with NR were prepared by the injection of a $(FFiK)_2$ solution into DMEM (Figure 8A). HepG2 cells were suspended with hydrogel fibers and cultured for 2 days. The hydrogel fibers maintained their structure as prepared under cell culture condition (37 °C, 5% CO_2) (Figure 8D). The HepG2 cells also adhered to the surface of $(FFiK)_2$ hydrogel fibers and proliferated (Figure 8B,C). This result shows that the shaped $(FFiK)_2$ hydrogel can be used as a cell culture scaffold.

4 | CONCLUSION

We designed new short self-assembling peptides, $(FFiO)_2$ and $(FFiK)_2$, with a symmetric structure via a urea bond. The simple structural design of the peptides makes their synthesis on a large scale possible. CD experiments revealed that both $(FFiO)_2$ and $(FFiK)_2$ assembled into β-sheet structures in a concentration-dependent manner at pH 7.2. $(FFiK)_2$ exhibited a self-assembling ability superior to that of $(FFiO)_2$, because $(FFiK)_2$ assembled at a lower concentration than $(FFiO)_2$. In addition, the self-assembly of $(FFiO)_2$ and $(FFiK)_2$ was dependent on pH. Electrostatic interactions of ornithine or lysine residues at the edge of both peptides contributed to their stable assembly in β-sheet structures under physiological pH condition. TEM observations revealed that both $(FFiO)_2$ and $(FFiK)_2$ assembled into nanofibers with a uniform width and length more than 500 nm at pH 7.2. $(FFiK)_2$ was present as more densely bundled and networked nanofibers than $(FFiO)_2$ due to its superior self-assembling ability. Nanostructures of $(FFiK)_2$ were also dependent on pH, as reflecting by the CD results. Electrostatic interactions at the edge of the peptides were important for their assembly into highly networked nanofibrous structures. Hydrogelation experiments revealed that both $(FFiO)_2$ and $(FFiK)_2$ formed transparent hydrogels at pH 7.2. $(FFiK)_2$ exhibited a lower critical hydrogelation concentration than $(FFiO)_2$, which is consistent with the CD results. $(FFiK)_2$ formed a more transparent hydrogel than $(FFiO)_2$; on the other hand, the Ac-FFiK peptide, which does not contain a urea unit, formed an opaque hydrogel at a high concentration. Not only the lysine residue but also the urea unit were important for the formation of transparent hydrogels. In addition, hydrogel fibers were prepared by the injection of a $(FFiK)_2$ solution at a high concentration into PBS or DMEM, because $(FFiK)_2$ exhibited rapid hydrogelation. In cell culture experiments, we successfully demonstrated that the $(FFiK)_2$ hydrogel could be used as a scaffold for the culture of HepG2 cells without significant cytotoxicity. The $(FFiK)_2$ hydrogel promoted the proliferation of HepG2 cells. The hydrogel fiber of $(FFiK)_2$ also worked as a scaffold for HepG2 cells. Based on these results, $(FFiK)_2$ is a beneficial supramolecular peptide hydrogelator with cell compatibility. Further functionalization of $(FFiK)_2$ will produce biomaterials useful for cell engineering and biomedical applications, because $(FFiK)_2$ contains two amino groups that can be conjugated with functional molecules.

Supporting information contains synthesis of Ac-FFiK, ATR-FTIR spectra, hydrogelation of Ac-FFiK, transparency measurement of hydrogels, cell culture on hydrogels (1 and 3 days), ^{1}H NMR, and MALDI-TOF MS of compounds.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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