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(Citation)

Langmuir, 34(27):8065-8074

(Issue Date)

2018-07-10

(Resource Type)

journal article

(Version)

Accepted Manuscript

(Rights)

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<https://hdl.handle.net/20.500.14094/90005027>



Short oligopeptides for biocompatible and biodegradable supramolecular hydrogels

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KEYWORDS. amino acids, cytotoxicity, encapsulation, self-assembly, short oligopeptide, stimuli-responsive hydrogels

ABSTRACT. Short Phe-rich oligopeptides, consisting of only four and five amino acids, worked as effective supramolecular hydrogelators for buffer solutions at low gelator concentrations (0.5–1.5 wt.%). Among ten different oligopeptides synthesized, peptide P1 (Ac-Phe-Phe-Phe-Gly-Lys) showed high gelation ability. Transmission electron microscopy observations suggested that the peptide molecules self-assembled into nanofibrous networks, which turned into gels. The hydrogel of peptide P1 showed reversible thermal gel-sol transition and viscoelastic properties typical of a

gel. Circular dichroism spectra revealed that peptide P1 formed a β -sheet-like structure, which decreased with increasing temperature. The self-assembly of peptide P1 occurred even in the presence of nutrients in culture media and common surfactants. *Escherichia coli* and yeast successfully grew on the hydrogel. The hydrogel exhibited low cytotoxicity to animal cells. Finally, we demonstrated that functional compounds can be released from the hydrogel in different manners based on the interaction between the compounds and the hydrogel.

Introduction

Supramolecular hydrogels, whose networks are constructed by noncovalent interaction between gelator molecules, are soft materials with a wide range of applications and have been energetically studied owing to their structural and functional properties.¹⁻⁵ Their wide potential applications proposed are therapeutic drug carriers, catalysts, media for organic/inorganic reactions and absorbents for pollutant removal from waste water.⁶⁻⁹ Supramolecular hydrogels are divided into two categories: hydrogels made of polymers with high molecular weights and those of low-molecular-weight hydrogelators.¹⁰⁻¹¹ For the last two decades, various kinds of functional low-molecular-weight hydrogelators have been reported because the molecular structures of low-molecular-weight hydrogelators can be precisely designed to exhibit the functional properties of the resultant hydrogels. Hydrogelators that are derived from peptides are especially of great importance because of their high biocompatibility.¹²⁻¹⁵ They are also studied as good model systems for the biological self-assembly (e.g., Alzheimer's A β peptide) in nature.¹⁶⁻¹⁷ The utilization of amino acids as a building block of a hydrogelator provides a facile pathway to produce supramolecular hydrogels with designable functions, which are different from those of

other organic/inorganic molecules.¹⁸ By designing the amino acid sequence, the secondary structure of peptides, such as β -sheet and α -helix, can be prepared to control the noncovalent interactions between peptides, including hydrophobic interactions, hydrogen bonding, π - π interactions, van der Waals interactions and electrostatic interactions.¹²⁻²¹ Some of the designed peptides self-assemble to form three-dimensional network structures and turn into hydrogels (peptide-based hydrogelators).¹¹⁻¹³ The noncovalent interactions between peptides can also be designed to be responsive to various external stimuli (heat, pH, light, ions, enzyme and small molecules), leading to stimuli-triggered hydrogelation and stimuli-triggered gel-sol transition.²²⁻³⁰

There are many examples of short-peptide-based hydrogelators.^{1,20,21} The shortest peptide hydrogelator composed of two amino acids, Ile-Phe, reported by Ventura *et al.*, which formed a hydrogel (only for water) at 1.5 wt.% through the formation of nanofibers with a diameter of approximately 55 nm.³¹ Hamley *et al.* designed a pentapeptide, Lys-Leu-Val-Phe-Phe, inspired from a fragment of the amyloid β peptide and succeeded in the hydrogelation of phosphate-buffered saline at a relatively high concentration (3.0 wt.%).³² Another oligopeptide reported by Lu *et al.* is a capped hexapeptide, Ac-Ile-Ile-Ile-Cys-Gly-Lys-NH₂, resulted in hydrogel formation at 0.53 wt.%, which was mechanically very weak.³³ Ulijn *et al.* proposed a computational approach to predict the self-assembly of numerous peptides and successfully found self-assembling tripeptides (Lys-Tyr-Phe, Lys-Tyr-Tyr and Lys-Phe-Phe) and tetrapeptide (Lys-Tyr-Phe-Trp) that formed hydrogels (for phosphate buffer) at 1.4 wt.%.³⁴ The conjugation of amino acid residues (or peptides) with long alkyl chains (e.g., palmitoyl group) or aromatic group (Fluorenyl and Naphthyl groups) is an efficient approach for the that preparation of supramolecular hydrogels with low concentrations.^{23,35-37} However, there are several reports that describe their remarkable toxicity to bacterial and animal cells.^{38,39}

For the application of the supramolecular hydrogels in biological and biomedical fields, the major requirements of the hydrogelators are biocompatibility, biodegradability, a low minimum gelation concentration (e.g. < 1 wt.% from the practical point of view) and no pathogenic contamination (often derived from animal resources). A peptide-based hydrogel has a high potential for biocompatibility and biodegradability. A chemically synthesized peptide minimizes the risk of pathogenic contamination. In this study, we found synthetic short oligopeptides with biocompatibility and biodegradability that self-assembled into nanofibrous networks to form hydrogels at low oligopeptide concentrations. Among 10 different types of oligopeptides synthesized, we found an N-acetylated tetrapeptide and N-acetylated pentapeptides that worked as effective supramolecular hydrogelators at 1.5 wt.% or less. Cytotoxicity studies revealed that one of the pentapeptides we found had low toxicity to animal and bacterial cells. The peptide hydrogelator could be hydrolyzed by a proteolytic enzyme (biodegradability). We also demonstrated that the peptide-based hydrogel, encapsulating functional compounds, showed different release profiles based on the interaction between the peptide and the encapsulated compounds.

Experimental

Materials

Fmoc-Lys(Boc)-Alko resin, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Phe-OH, Ac-Phe-OH, *O*-(1H-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxy-1H-benzotriazole hydrate (HOBt.H₂O), triisopropylsilane (TIPS) and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). *N,N*-Diisopropylethylamine (DIEA) was purchased from

Tokyo Chemical Industry (Tokyo, Japan). Kaiser reagents for ninhydrin tests were purchased from Kokusan Chemical (Tokyo, Japan). Cell counting kit-8 was purchased from Dojindo Molecular Laboratories, Inc. (Kumamoto, Japan). Fetal bovine serum (FBS) was purchased from Sigma Aldrich, Inc. (St. Louis, MO, USA). Dulbecco modified eagle medium (DMEM), penicillin-streptomycin and trypsin were purchased from Nacalai Tesque (Kyoto, Japan). Enkephalin and doxorubicin were purchased from LKT Laboratories, Inc. (St Paul, MN) and Oakwood Products, Inc. (Estill, SC, USA), respectively. Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Water used was produced with a Millipore water purification system. All compounds were used without further purification.

Synthesis and purification of peptides

Peptides were synthesized on Fmoc-Lys(Boc)-Alko resin using the standard Fmoc solid-phase peptide synthesis method. A Fmoc-protected amino acid (3 equivalents) was coupled to 0.3 mmol amino-acid-preloaded Alko resin, using HBTU and HOBt as the coupling agents in the presence of DIEA in DMF. Qualitative ninhydrin tests were used to confirm the completion of each coupling reaction. The crude peptides were cleaved manually from the solid support and the side chains were deprotected using a TFA/TIPS/water (95 : 2.5 : 2.5 in volume) mixture at room temperature for 2 h. The crude peptides were precipitated and washed three times in diethyl ether and centrifuged at 7000 rpm for 5 min. The precipitates were lyophilized overnight. A high-performance liquid chromatography (HPLC) system, Shimadzu LC-20AT equipped with a UV-vis detector SPD-20A, was used for the purification of all crude peptides. For the purification, solvent A consisting of 0.1% TFA in water and solvent B consisting of 0.1% TFA in acetonitrile were used as eluents. A linear gradient of 0–100% B over 20 min was applied. The final compound

was characterized by a matrix-assisted laser desorption ionization–time of flight mass spectrometer (MALDI-TOF/MS) measurements using an UltrafleXtreme™ mass spectrometer (Bruker, Billerica, MA) (MS data were in SI). The purified peptides were obtained after lyophilization of the collected HPLC fractions. The obtained peptides were stored at -4 °C until use.

Preparation of peptide hydrogels

Each peptide was typically placed separately in a glass tube ($d = 8$ mm) and was dissolved in an aqueous buffer solution and water by heating on a sand bath at 85 °C followed by ultrasonication for 5 min. Heating at 85 °C was to make sure to dissolve all the gelators in aqueous solutions. Then, the solutions were slowly cooled down to room temperature to form a hydrogel. Gelation was confirmed by inverting the glass tube containing the solution. Phosphate, HEPES and Tris-HCl buffers (50 mM, pH 7.4) and water were used for hydrogel preparation.

Transmission electron microscope (TEM) observations

Carbon-coated copper grids (ELS-C10, Okenshoji Co., Ltd., Tokyo, Japan) were used for TEM observations. A drop of a hydrogel sample was pipetted onto a grid, followed by staining with aqueous potassium phosphotungstate (2 wt.%). The grids were vacuum-dried. The samples were observed using a JEOL JEM-2100 F transmission electron microscope (Tokyo, Japan) at an operating voltage of 200 kV.

Circular dichroism (CD) spectroscopy analysis

The secondary structure of the peptides was analyzed using a 0.1 cm quartz cell on a Jasco J-725K spectropolarimeter with 4 s integrations, 4 accumulations and a step size of 1.0 nm, with a bandwidth of 1.0 nm over a range of wavelengths from 200 to 250 nm. A 25 mM phosphate buffer solution containing a peptide (0.05 wt%) was put in a quartz cell and was left for 1 h. Spectra were recorded at several temperatures (25–90 °C).

Rheology measurements

Rheology measurements were carried out using a rheometer (Anton Paar Physica MCR301, Graz, Austria) with a parallel plate (diameter = 5.0 cm) at a strain of 0.1 % and a gap of 0.3 mm. For sample measurements, hydrogels at their MGCs were loaded on a sample plate whose temperature was set at 90 °C. The sample plate was then gradually cooled to 25 °C (more than 30 min) and the measurement was started.

Differential scanning calorimetry (DSC) measurements

The gel-sol transition temperatures (T_{gel}) of the hydrogels were measured using a differential scanning calorimeter (DSC7000X, Hitachi, Tokyo, Japan) in aluminum pans with a heating rate of 2 °C/min.

Cytotoxicity study

HeLa cells and NIH3T3 cells were cultured in DMEM solutions supplemented with 10% (v/v) FBS, 1 U/ml penicillin and 1 µg/ml streptomycin using a 96-well microplate in 5% CO₂ atmosphere at 37 °C. The culture medium was exchanged every 72 h with a fresh one, and cells were harvested with phosphate-buffered saline (PBS) containing 0.25 g/l trypsin and counted using

a cell counter (Cell Counter model R1, Olympus, Tokyo, Japan). The resulting cell suspension was diluted in a DMEM solution and the cells were seeded at a density of 5000 cells per well in a 96-well microplate followed by culturing for 24 h. Then, the culture medium was exchanged with DMEM medium containing 10% (v/v) FBS and a hydrogelator at a given concentration in a 96-well microplate (100 μ L). After the cells were cultured for 24 h, the viability of the cells was determined using Cell counting kit-8.

Enzymatic hydrolysis of a hydrogel

α -Chymotrypsin was used for the hydrolysis of the peptides. A stock solution (0.02 wt.% chymotrypsin in 20 mM HCl aqueous solution) was prepared. A hydrogel (250 μ L) containing 1.0 wt.% peptide P1 was prepared using a phosphate buffer solution (pH 7.4, 0.1 M) in a glass vial and the enzyme stock solution (25 μ L) was added, followed by incubation at 40 °C. A fresh phosphate buffer solution (250 μ L, pH 7.4, 0.1 M) was added to the glass vial and shaken vigorously to dissolve the hydrogel. Aliquots (100 μ L) were periodically taken at 0, 1, 2, 3 and 9 h and analyzed by HPLC. As a control, 20 mM HCl aqueous solution (25 μ L) was added to the hydrogel instead of an enzyme stock solution.

Encapsulation of functional compounds and their release from a hydrogel

A hydrogel of peptide P1 (1.0 wt.%, 250 μ L) was prepared using a phosphate buffer solution (pH 7.4, 0.1 M) containing 0.05 wt.% functional compound. A phosphate buffer solution (500 μ L, pH 7.4, 0.1 M) was gently added on top of a hydrogel without disrupting the hydrogel. Aliquots (100 μ L) were taken periodically from the supernatant and analyzed by HPLC. The released

compounds were measured by the absorbance (215 nm for uranine, 224 nm for enkephalin and 233 nm for doxorubicin).

Results and discussion

Peptide design and self-assembly

Many of the previous reports on peptide-amphiphile hydrogelators suggest the importance of the hydrophobic moiety in the hydrogelator and the balance between the hydrophilic and hydrophobic moieties.¹²⁻¹⁴ These lead into the self-assembly process in hydrogelators and their gelation ability. Xu *et al.* demonstrated that oligophenylalanine worked as a hydrophobic moiety to produce a hydrogelator composed only of a peptide.⁴⁰ Zhang *et al.* reported that Lys-terminated long oligopeptides can self-assemble to form nanostructures.⁴¹ We adopted a sequence of Phe residues as a hydrophobic moiety and a Lys residue at a C-terminus to induce molecular self-assembly. We synthesized 10 different types of oligopeptides composed of four and five amino acids. The design of the short oligopeptides and the results of the gelation tests are summarized in Table 1. We employed 3 different kinds of aqueous buffers (50 mM, pH 7.4) and water for the hydrogelation because molecular self-assembly of a peptide is often influenced by the buffer salts.⁴²⁻⁴⁴ Among the 10 synthesized oligopeptides, peptides P1-P4 (pentapeptides and tetrapeptide) successfully gelled the phosphate buffer, HEPES buffer, Tris-HCl buffer and water at a concentration below 1.5 wt.%. Peptide P5 also formed a hydrogel only in the phosphate buffer at 0.8 wt.%. Figure 1 shows the appearance of the hydrogels using a phosphate buffer and peptides. Peptide P1 gave a translucent hydrogel, indicating a thin fibrous network of the hydrogels. Peptides P4 and P5 gave heterogeneous hydrogels. In particular, peptide P1 produced hydrogels of all the buffers tested and

water only at 0.5 wt.%. Interestingly, the minimum gelation concentrations (MGCs) of these peptides varied with the buffers.

The sequence of amino acids in a peptide usually plays an important role in its self-assembly.⁴⁵⁻⁴⁷ Peptides P1-P4 had 3 repeated Phe and Lys residues at the C-termini. Their N-termini were acetylated. There were differences in the amino acids between the Phe and Lys residues in their sequences. Although Val and Ala residues have often been used in peptide-amphiphile hydrogelators reported previously,⁴⁵⁻⁴⁷ the exchange of Gly with Val or Ala residues resulted in a low gelation ability. Although the deletion of the Gly residue also reduced the gelation ability (peptide P4), the acetylated short peptide, tetrapeptide, can gelate HEPES buffer only at 1.0 wt.%. In our previous study, we revealed that the Gly residue strengthened the interaction between peptide amphiphiles; therefore, the Gly residue also played an important role for the self-assembly of peptide P1.²³

Table 1. Gelation properties of synthesized oligopeptides.

Peptide	Amino acid sequences	Gelation test in			
		Phosphate buffer	Tris-HCl buffer	HEPES buffer	Water
P1	Ac-Phe-Phe-Phe-Gly-Lys-OH*	G (0.5) **	G (0.5)	G (0.5)	G (0.5)
P2	Ac-Phe-Phe-Phe-Val-Lys-OH	G (1.5)	G (1.5)	G (1.0)	G (1.5)
P3	Ac-Phe-Phe-Phe-Ala-Lys-OH	G (1.5)	G (1.0)	G (1.5)	G (1.5)
P4	Ac-Phe-Phe-Phe-Lys-OH	G (1.5)	G (1.5)	G (1.0)	G (1.5)
P5	Ac-Phe-Phe-Phe-Gly-Lys-NH ₂	G (0.8)	S	S	S
P6	H-Phe-Phe-Phe-Gly-Lys-NH ₂	S	S	S	S
P7	H-Phe-Phe-Phe-Gly-Lys-OH	S	S	S	S

P8	Ac-Phe-Phe-Gly-Lys-OH	S	S	S	S
P9	Ac-Phe-Phe-Val-Lys-OH	S	S	S	S
P10	Ac-Phe-Phe-Ala-Lys-OH	S	S	S	S

*Ac represents an acetyl group. **G and S indicate gel and solution, respectively. Minimum gelation concentrations (MGCs) in wt.% are given in parentheses. All buffers are 50 mM and at pH 7.4. Gelation tests were carried out using peptides at a concentration of less than 2.0 wt.%.

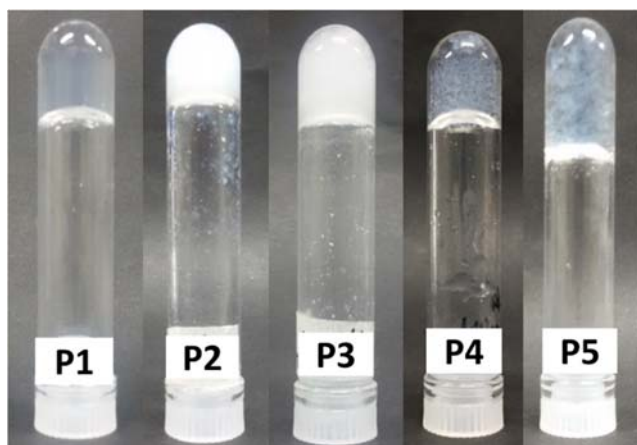


Figure 1. Hydrogels prepared with peptide P1-P5 in 50 mM phosphate buffer solution (pH 7.4) at their MGCs.

Peptides P5, P6 and P7 were designed to study the influence of the charges on the C-terminus and N-terminus of peptide P1 (Table 1). Peptide P5, which had an amide at its C-terminus, gelled only phosphate buffer at 0.8 wt.% (Figure 1) and did not gelate other kinds of aqueous buffer. The amidation of the C-terminus reduced the gelation ability. Peptides P6 and P7 did not harden any aqueous solution and they were soluble in these solutions at 2.0 wt.%. The protonation of amino groups at the N-termini of the peptides would contribute to the dissolution of peptides and prevent their self-assembly. Indeed, peptides P6 and P7 were not soluble in aqueous buffer at pH 10. The influence of the acetyl group agreed with the results reported by Ballet *et al.*, which showed the

addition of an acetyl group at the N-termini of the peptides led to aggregation in aqueous solution.⁴⁶ The electrostatic charges of the C- and N-termini were important in the self-assembly of peptides and hydrogelation.

To investigate the effect of the Phe residues, we designed peptides P8-P10, which contained only 2 Phe residues. The gelation tests showed that they were soluble in aqueous solutions at 2 wt.%, meaning no hydrogelation occurred. This might be owing to insufficient hydrophobicity and insufficient π - π interactions between peptide molecules.

Peptide self-assembly in the presence of surfactants

Since some surfactants have functional properties (*e.g.*, antibacterial activity and vesicle formation), the combination of supramolecular hydrogels and surfactants provides novel soft materials that integrating multiple functions. Although common surfactants inhibit self-assembly of peptide hydrogelators, van Esch *et al.* reported orthogonal self-assemblies of a supramolecular hydrogelator and surfactants. They succeeded in the preparation of a fibrous network formed by a supramolecular hydrogelator and vesicles formed by a surfactant to create a highly complexed self-assembled objects.⁴⁸ Because our present hydrogelator (peptide P1) was composed only of amino acids, we expected less interaction between peptide P1 and surfactants. The gelation tests of peptide P1 were carried out by dissolving peptide P1 (1.0 wt%) in a phosphate buffer containing each surfactant (anionic, cationic and nonionic ones) with heating and ultrasonication (Figure 2). After cooling to room temperature, peptide P1 (1.0 wt%) successfully gelled phosphate buffer and water in the presence of 0.5 wt.% nonionic (brij 35) and anionic surfactants (tween 20 and sodium dodecyl sulfate). Those concentrations (0.5 wt.%) of nonionic and anionic surfactants were above their critical micelle concentrations (0.011 wt.% for brij 35, 0.0074 wt.% for tween 20 and

0.23 wt.% for sodium dodecyl sulfate). In the presence of 0.5 wt.% cationic surfactants (benzalkonium chloride and cetylpyridinium chloride), which was above their critical micelle concentrations (0.01 wt.% for benzalkonium chloride and 0.004 wt.% cetylpyridinium chloride), the gelation with peptide P1 occurred only in phosphate buffer and not in water, probably owing to the change of pH in water, which turns acidic. There were small differences in the appearance of the hydrogel in the presence and absence of surfactants. These results indicated that the self-assembly of peptide P1 occurred even in the presence of these surfactants, suggesting that the hydrophobic interactions did not play a major role in the self-assembly of peptide P1. The results also indicated the potential application of the combination of the hydrogel and a surfactant (e.g., a hydrogel with an antiseptic surfactant).

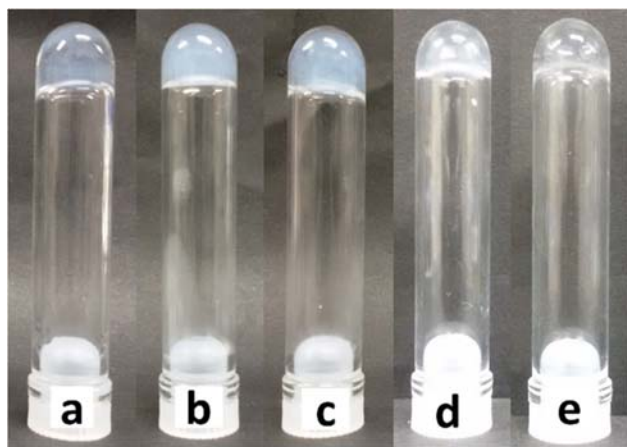


Figure 2. Hydrogel of 50 mM phosphate buffer (pH 7.4) at 1.0 wt.% peptide P1 in presence of 0.5 wt.% surfactant. (a) Tween 20, (b) benzalkonium, (c) brij 35, (d) benzalkonium chloride and (e) cetylpyridinium chloride.

TEM observations

To obtain morphological information of the hydrogels prepared with peptides P1-P4, TEM observations were carried out (Figure 3). We observed nanofibrous networks of these hydrogels, similar to other low-molecular-weight hydrogelators.^{23,28-30} The peptide gelator molecules would self-assembled through noncovalent interactions such as π - π interactions, hydrophobic interaction and/or hydrogen bonding to form entangled nanofibers. The repeated phenylalanine residues would provide hydrophobic interaction and π - π interactions among the peptide molecules in the solvent. The images of the nanofibrous network structures of all the hydrogels showed a width of approximately 30-60 nm and they extended to several micrometers in length. The difference in peptide sequences resulted in a variety of the formed nanofibrous structures. The hydrogel of P1 showed the branched nanofibrous structure. Hydrogels with peptides P2 and P3 seemed to form long and straight nanotubular structures and to have relatively few entanglements and branching, which might lead to the relatively high MGCs. These morphological differences would be derived from the different intermolecular interactions (hydrogen bonding, etc.) of peptide sequences and the different packing configurations of the peptide molecules. The hydrogel of peptide P4 showed relatively short fibers (less than 1 μ m) and less crosslinked fibrous network. Although there were only small differences in the peptide sequences, a wide variety of nanofibrous morphology were obtained.^{49, 50}

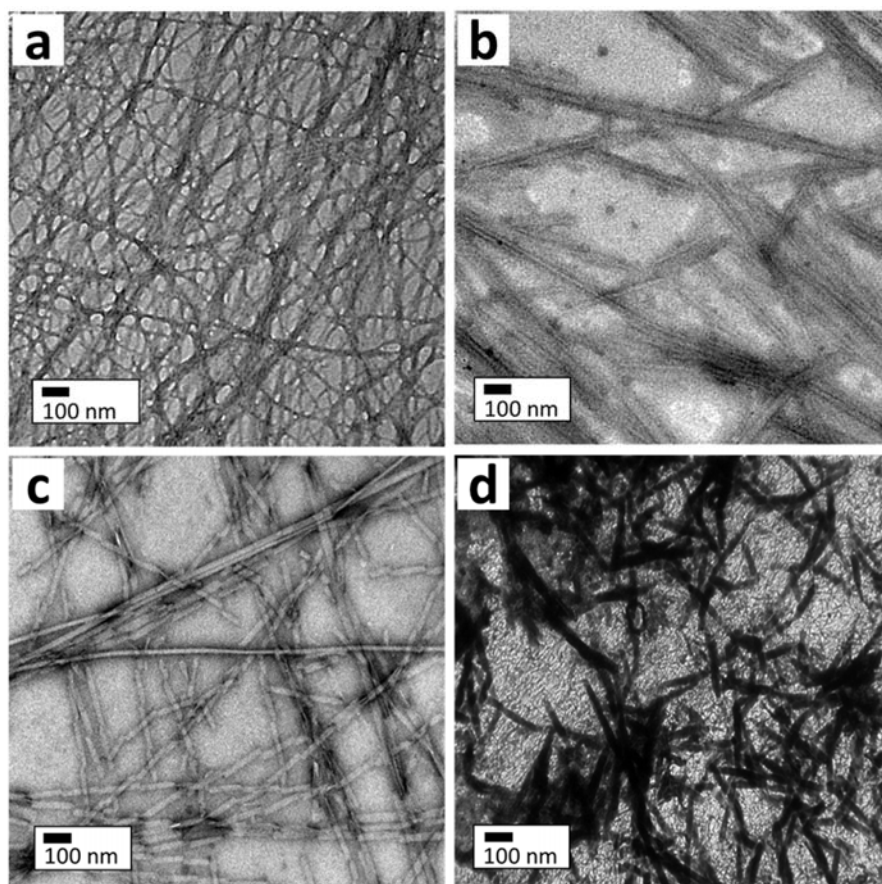


Figure 3. TEM images of hydrogels in 50 mM phosphate buffer (pH 7.4) at their MGC. (a) P1 hydrogel, (b) P2 hydrogel, (c) P3 hydrogel and (d) P4 hydrogel.

Rheological and calorimetry measurements

Rheological experiments were performed to characterize the hydrogels prepared with peptides P1-P4. The storage modulus (G') and loss modulus (G'') were measured in frequency sweep experiments at a fixed strain of 0.1% and are shown in Figure 4. For all the hydrogels tested, G' were higher than G'' , indicative of a dominant elastic behavior such as the formation of a stable gel.^{23,51,52} The hydrogel of peptide P1 showed G' that was seven-fold higher than G'' . Hydrogels of peptides P2, P3 and P4 exhibited G' that were three-, five- and six-fold higher than G'' ,

respectively. Each hydrogel exhibited different elasticity. This could be correlated with the fibrous networks composed of peptides and the intermolecular interactions between peptides. For example, the hydrogel of peptide P1 exhibited the highest G' among the hydrogels and peptide P1 formed finest nanofibers among the peptides. TEM images showed that nanofibers of peptide P1 seemed elastic and that those of peptides P2 and P3 seemed straight and robust. For the hydrogel of peptide P1, the strain-sweep measurements were also carried out with increasing strain from 0.1 – 1.0 %. We observed that G' and G'' were constant over the tested range and that G' was seven or eight times higher than G'' over the tested range (Figure S1).

The gel-sol transition temperatures (T_{gel}) were measured by DSC. The hydrogel prepared with 0.5 wt.% peptide P1 showed an endothermic peak at 72 °C (Figure 5), whereas the hydrogel of 2.0 wt.% peptide P1 showed an endothermic peak at 87 °C. Observations by a naked eye also confirmed that the hydrogels with peptide P1 turned into solution above these temperatures upon heating. The T_{gel} of the present hydrogel was affected by the gelator concentration.⁵³

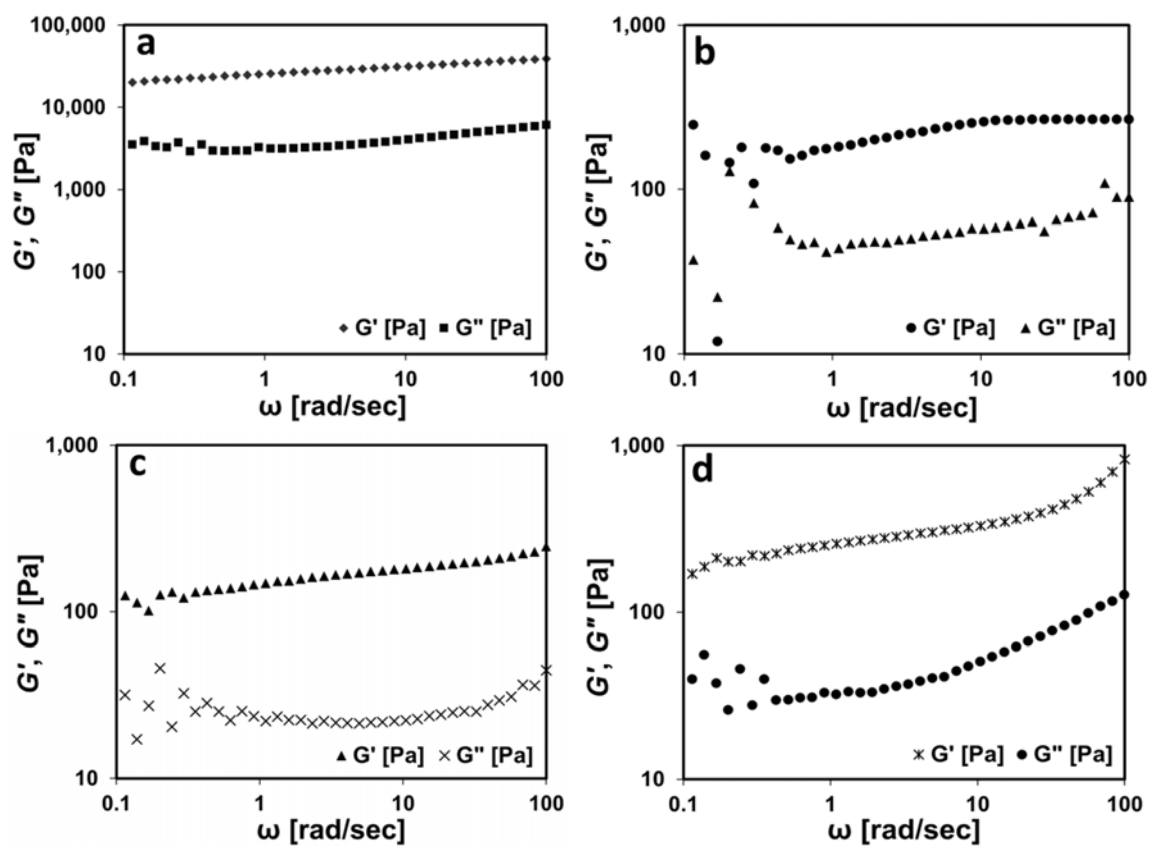


Figure 4. Rheological properties of hydrogels in 50 mM phosphate buffer solution pH 7.4 at their MGC. (a) P1 hydrogel, (b) P2 hydrogel, (c) P3 hydrogel and (d) P4 hydrogel.

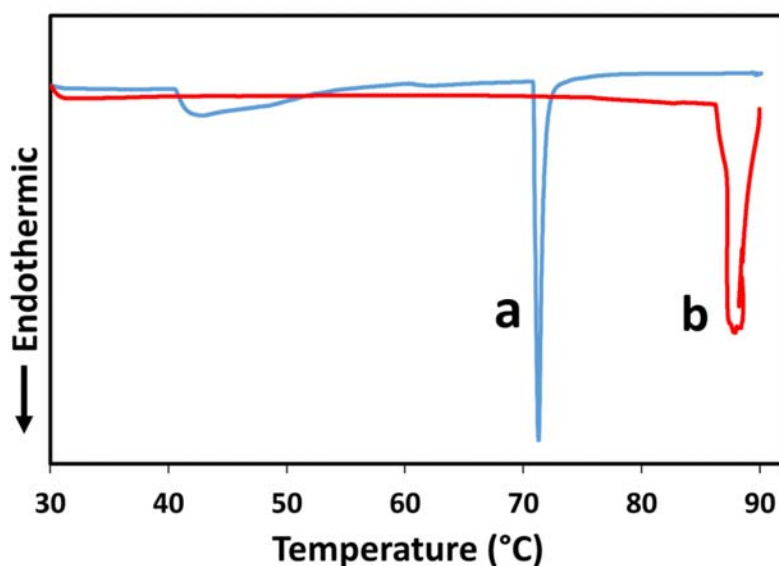


Figure 5. Calorimetric measurements of P1 hydrogels of 50 mM phosphate buffer (pH 7.4). (a) 0.5 and (b) 2.0 wt.% peptide P1.

Characterization of the secondary structure of peptide P1

To obtain the secondary structure information of the self-assembly of peptide P1, CD spectra were measured at various concentrations and different temperatures. Figure 6a showed that peptide P1 at its MGC (0.5 wt.%) and the concentration near MGC (0.4 wt.%, exhibiting a partial gel) had negative bands around 225 nm at 25 °C, indicating a β -sheet-like structure. This is in accordance with the hexapeptide hydrogelator reported by Ballet *et al.*,⁵⁴ in which the hydrogel exhibited β -sheet arrangements of the peptide in a gel state. Low concentrations of peptide P1 (0.1 – 0.3 wt.%) resulted in positive bands, meaning no formation of a β -sheet-like structure. These suggest that the concentration close to its MGC was critical for the self-assembly of peptide P1. Figure 6b showed the effect of temperature on the CD spectra of peptide P1 at 0.4 wt.%. When the temperature increased to 50 °C, the band around 225 nm became small. At 70 and 90 °C, the band turned

positive. These results indicate that the secondary structure of the peptide self-assembly turned from a β -sheet-like structure to random coils as the temperature increased.

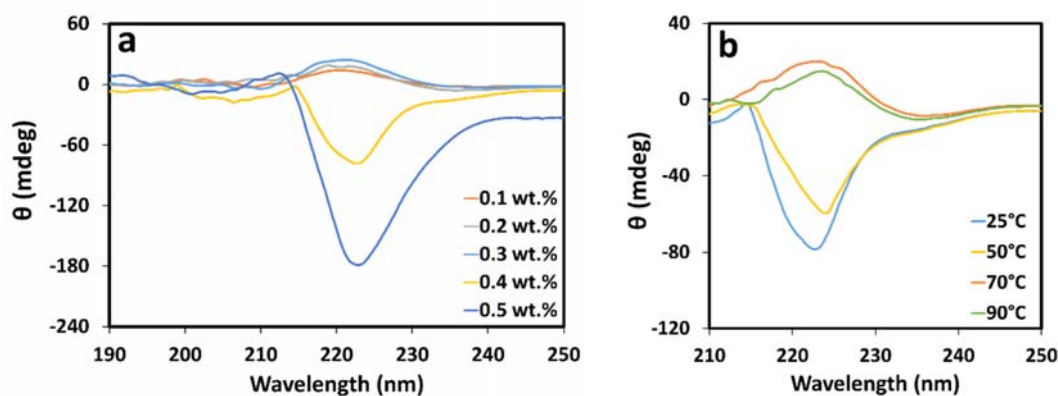


Figure 6. CD spectra of a 25 mM phosphate buffer solution containing peptide P1. (a) Various concentrations at 25 °C. (b) At various temperatures (0.4 wt.%).

Hydrogel as culture medium

One of the potential applications of supramolecular hydrogels is a scaffold for living cells.⁵⁵⁻⁵⁷ We attempted the gelation of lysogeny broth (LB) medium and yeast extract peptone dextrose (YPD) medium. LB medium is widely used for the growth of bacteria and contains tryptone at a relatively high concentration (1.0 wt.%), which is a mixture of peptides formed by the enzymatic digestion of casein. YPD medium is a complete medium for yeast growth and also contains a mixture of peptides at a relatively high concentration (2 wt.%). Both culture media were successfully gelated with 0.5 wt.% peptide P1, resulting in translucent hydrogels (Figure S2).

We then tried to culture *E. coli* and *Saccharomyces cerevisiae* (yeast) on the culture media gelated with peptide P1. *E. coli* successfully grew, which was comparable to that on a conventional agar medium (Figure S3). *S. cerevisiae* also grew on the YPD medium gelated with peptide P1, similar to that of the conventional agar medium (Figure S4). These results indicated that the culture

media gelled with peptide P1 were available for culturing of microorganisms and also imply the biocompatibility of P1 hydrogel.

The culture of HeLa cells were attempted on the hydrogel (DMEM medium + FBS) with 1.0 wt.% peptide P1 but the hydrogel was decomposed within 24 h at 37 °C. This decomposition would be induced by the proteolytic enzymes that were present in FBS or/and was secreted by HeLa cells. Indeed, the P1 hydrogel of DMEM medium (without FBS) with HeLa cells was stable more than 3 days (no obvious decomposition) and HeLa cells survived on the hydrogel (Figure S5). A part of the hydrogel of DMEM medium with FBS (without HeLa cells) was decomposed after 24 h incubation at 37 °C.

Cytotoxicity study

Although there are several reports of low-molecular-weight hydrogelators that induce cell death or inhibit bacterial growth,^{29,58-61} the above investigations on the microbial culture suggested the potential application of hydrogels in cell cultures and in the biomaterial field.^{55,62} Therefore, we studied the cytotoxicity of peptide P1 to animal cells. HeLa cells (human cancer cells) and NIH3T3 cells (mouse embryonic fibroblast cells) were used. As the concentration of peptide P1 was increased, the cell viability slightly decreased (Figure 7). Both cell lines retained more than 60 % viability even at 0.6 wt.% peptide P1, which exceeded the MGC of peptide P1. At 0.8 wt.%, the viability of NIH3T3 still remained over 60%. Although there are several studies that have described remarkable cytotoxicity of peptide-based hydrogelators to animal cells at low concentrations,^{29,60,61} the hydrogel of peptide P1 had low cytotoxicity to animal cells even above its MGC, probably because peptide P1 was composed of amino acids residues (not long fatty acid or polycyclic aromatic moieties).

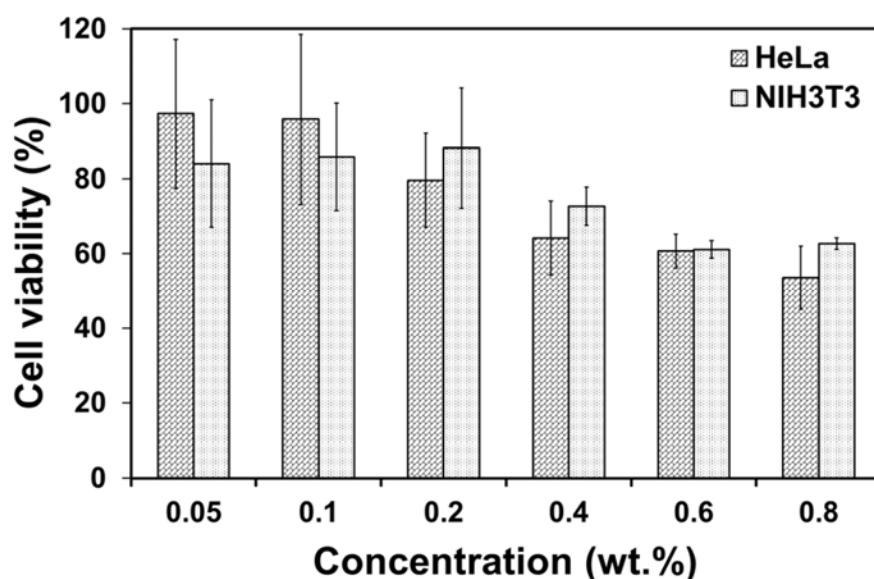


Figure 7. Cell viability of HeLa and NIH3T3 cells after 24 h incubation with peptide P1.

Enzymatic hydrolysis

One of the major advantages of a peptide is its susceptibility to enzymatic reactions. We studied the enzymatic degradation of the hydrogel of peptide P1 using α -chymotrypsin. Peptide P1 was composed of five L-amino acid residues, three of which were L-phenylalanine residues. In general, α -chymotrypsin catalyzes proteolysis and preferentially cleaves peptide bonds formed by aromatic residues (e.g. tyrosine, tryptophan, and phenylalanine). The HPLC analysis (Figure S6) revealed that the hydrolysis proceeded with time and that approximately 100 % conversion was obtained in 9 h (Figure 8). The degradation of the hydrogel was also confirmed by naked-eye observation (inset of Figure 8). The gel phase gradually turned to a sol phase as the hydrolysis proceeded. These results demonstrated the high biodegradability of the hydrogel of peptide P1.

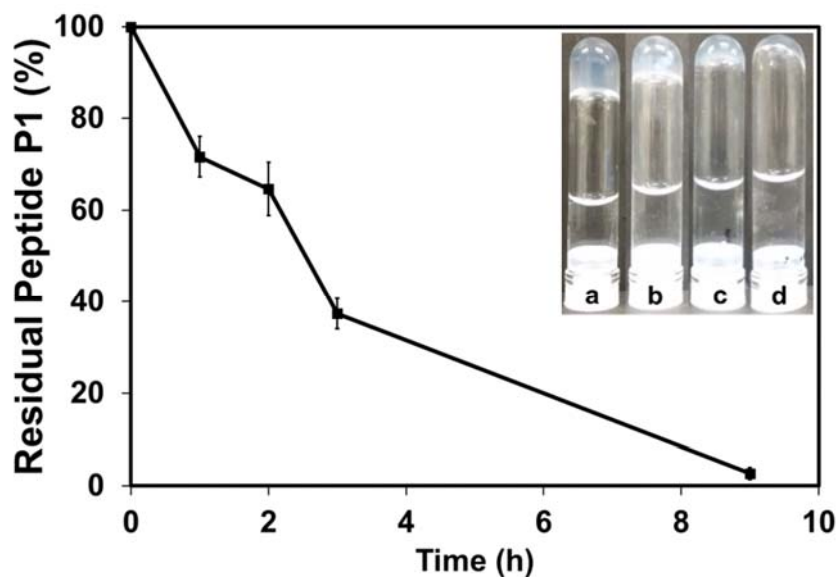


Figure 8. Enzymatic hydrolysis of the hydrogel of peptide P1 by α -chymotrypsin. The inset represents photos of P1 hydrogels during hydrolysis. (a) 0 h, (b) 1 h, (c) 3 h and (d) 9 h after the addition of α -chymotrypsin.

Release of functional compounds from a hydrogel

Several studies have reported that hydrogels of low-molecular-weight gelators could encapsulate and release functional compounds.^{45,46,63,64} We chose uranine, Leu-enkephalin (opioid peptide, YGGFL) and doxorubicin (anticancer agent) as model substrates and encapsulated these compounds in a hydrogel prepared with peptide P1. Figure 9a shows that one-third of uranine and enkephalin were released in the first 6 h, indicating less interaction between these compounds and the hydrogel. Although Leu-enkephalin is also a pentapeptide containing aromatic amino acids, the release results implied that Leu-enkephalin was not incorporated into the self-assembly of peptide P1.

A different release profile was observed for doxorubicin. Doxorubicin is a polyaromatic compound and is relatively hydrophobic. The released doxorubicin was less than 5 % even after 72 h. The polyaromatic structure or hydrophobicity of doxorubicin would strengthen the interaction with the self-assembly of peptide P1.⁶⁵ We then carried out the enzymatic hydrolysis of the hydrogel encapsulating doxorubicin to promote its release. Figure 9b shows the release of doxorubicin from the hydrogel in the presence of α -chymotrypsin. The hydrolysis of peptide P1 drastically expedited the release of doxorubicin from the hydrogel.

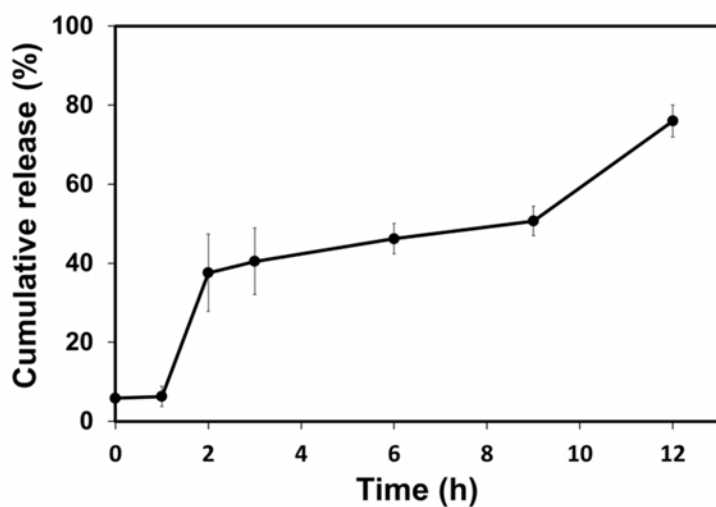
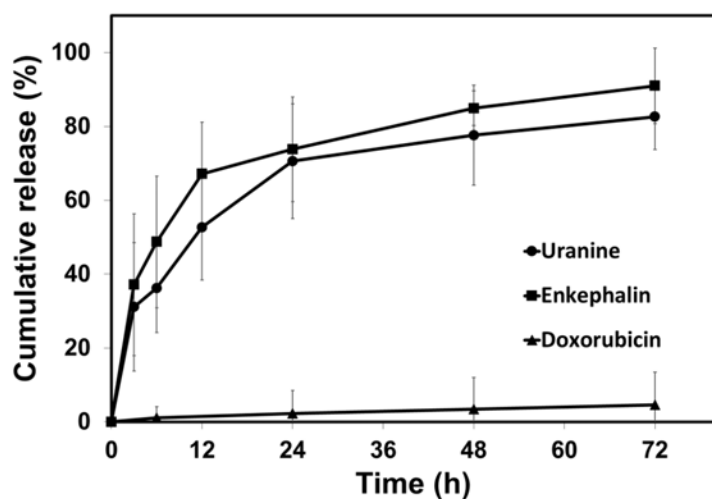


Figure 9. a) Release of functional compounds from P1 hydrogels. b) Release of doxorubicin promoted by enzymatic hydrolysis of P1 hydrogel. α -Chymotrypsin was used for the hydrolysis.

Conclusions

In summary, we synthesized 10 different types of simple and short oligopeptides (tetra- and pentapeptides) and found that 5 oligopeptides successfully gelled three different kinds of aqueous buffer solutions at physiological pH at relatively low concentrations. In particular, peptide P1 at concentrations as low as 0.5 wt.% can gelate buffer solutions. The rheological properties showed the gel-like properties. The hydrogel showed thermoreversible sol-gel transition and T_{gel} was influenced by the peptide concentration. TEM observations demonstrated the formation of entangled nanofibrous networks and CD measurements revealed that the β -sheet-like structure formed in the self-assembly of peptide P1. The self-assembly of peptide P1 occurred even in the presence of conventional surfactants. Peptide P1 had a low cytotoxicity to yeast, bacteria and animal cells, probably because it consisted only of amino acid residues. The hydrogel of peptide P1 was degraded by a proteolytic enzyme, showing its high biocompatibility. The high biocompatibility might be related to the low cytotoxicity of peptide P1. The hydrogel encapsulating functional compounds released them in different manners depending on the interactions with the self-assembly of peptide P1. These characteristics of the present hydrogel promise the integration of other functional substances (e.g., an antiseptic surfactant, lipid vesicles, a functional peptide, functional proteins, surfactant-coated nanomaterials and cells) to create multifunctional hydrogels available for the fields of bioengineering, tissue engineering and drug delivery.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge (PDF). The rheological properties of P1 hydrogel in amplitude sweep and CD spectra of peptide P7. The results of gelation tests for culture media. Cultivation of microorganisms and HeLa cells on the hydrogels. HPLC results of enzymatic hydrolysis of peptide P1 hydrogel.

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Author Contributions

T.M. designed the project. W.K.R. and Y.N., synthesized the peptides and carried out fundamental characterization of the peptides. W.K.R. and S.Y. did spectroscopic analysis, microscopy observations and cytotoxicity tests. W.K.R. and J. I. cultured microorganisms. W.K.R., J.I. and T.M. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Funding Sources

This study was financially supported by Tokuyama Science Foundation, by Takeda Science Foundation, by the Special Coordination Funds for Promoting Science and Technology, Creation of Innovation Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe), MEXT, Japan, by JSPS KAKENHI Grant Numbers 16H04577 & 18H04556, and also by the Japan Science and Technology (JST) and Japan International Cooperation Agency (JICA), Science and Technology Research Partnership for Sustainable Development (SATREPS), and Ministry of Research, Technology and Higher Education of Indonesia on Research and Innovation in Science and Technology Program (RISET-PRO).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Prof. H. Minami, Prof. A. Kondo, Prof. H. Suzuki and Prof. Y. Komoda for technical helps with DSC, MALDI TOF/MS and a rheometer, respectively. We thank Zoran Dinev, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

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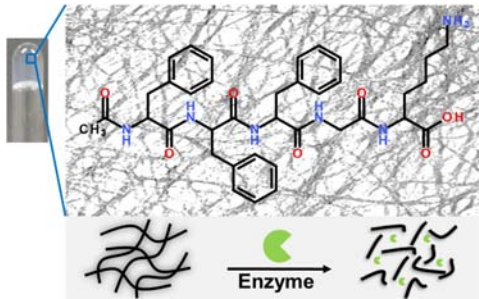
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TOC



Short oligopeptides for biocompatible and biodegradable supramolecular hydrogels

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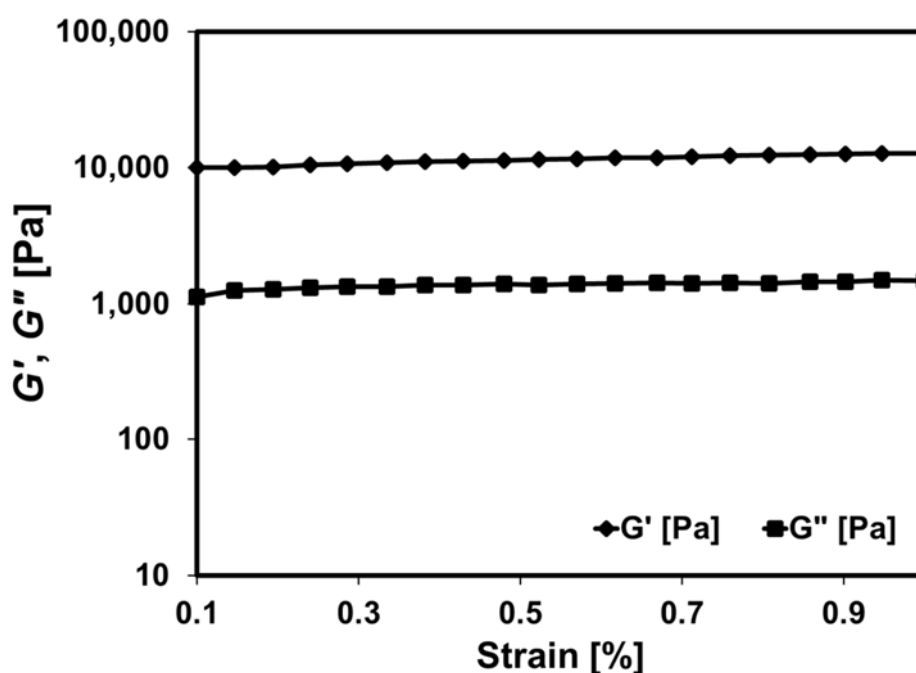


Figure S1. Rheological properties of the hydrogel prepared with 0.5 wt.% peptide P1 in 50 mM phosphate buffer solution (pH 7.4).

Preparation of peptide hydrogels

LB medium was prepared by mixing 0.5 wt.% yeast extract, 1.0 wt.% tryptone and 1.0 wt.% sodium chloride. YPD medium was prepared by mixing 1.0 wt.% yeast extract, 2.0 wt.% peptone and 2.0 wt.% D-glucose. Peptide P1 was placed in a glass tube (d = 8 mm) and was dissolved in LB or YPD medium by heating on a sand bath at 85 °C followed by ultrasonication for 5 min. The solutions were slowly cooled down to room temperature to form a hydrogel.

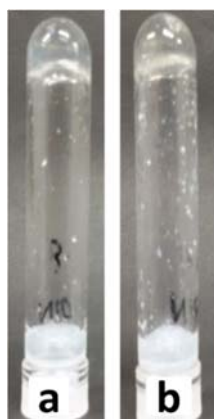


Figure S2. Gelation tests of (a) LB and (b) YPD media with 0.5 wt.% peptide P1.

Preparation of a peptide hydrogel as growth medium for *E. coli*

A hydrogel medium was prepared by mixing 0.5 wt.% yeast extract, 1.0 wt.% tryptone, 1.0 wt.% sodium chloride and 1.0 wt.% peptide P1 in water solution and followed by autoclave. A mixture solution (500 µl) was placed on a glass petri dish and left overnight at room temperature to form a hydrogel.

Culture of bacteria on hydrogels

E. coli MG1655 was inoculated in the LB medium overnight and 37 °C in a tube-shaker incubator and was calculated to reach the optical density (OD) of 0.9-1.0. A bacteria suspension (50 µl) was

placed on a hydrogel medium that was prepared a day before. The growth of bacteria at 37 °C was observed after 24 h.

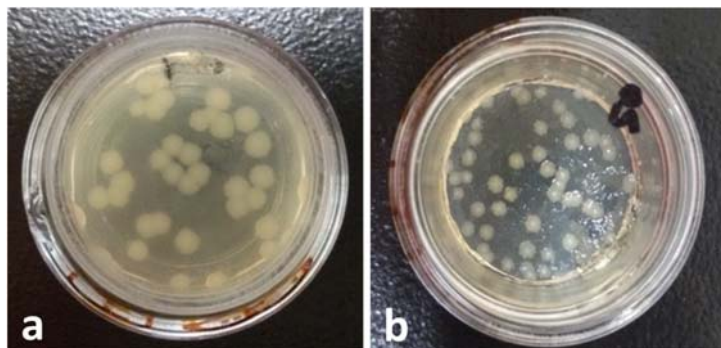


Figure S3. Cultivation of *E. coli* (MG1655) on LB media containing (a) 1.5 wt.% agar and (b) 1.0 wt.% peptide P1.

Preparation of a peptide hydrogel as growth medium for yeast

A hydrogel medium was prepared by mixing 1.0 wt.% yeast extract, 2.0 wt.% peptone, 2.0 wt.% D-glucose and 1.0 wt.% peptide P1 in water and followed by autoclave. A mixture solution (500 μ l) was placed in a glass petri dish and left overnight at room temperature to form a hydrogel.

Culture of yeast on hydrogels

Saccharomyces cerevisiae BY4741 was inoculated in the YPD medium overnight at 30 °C in a tube-shaker incubator and was calculated to reach the optical density (OD) of 0.9-1.0. A yeast suspension (50 μ l) was placed on a hydrogel medium that was prepared a day before. The growth of yeast at 30 °C was observed after 72 h.

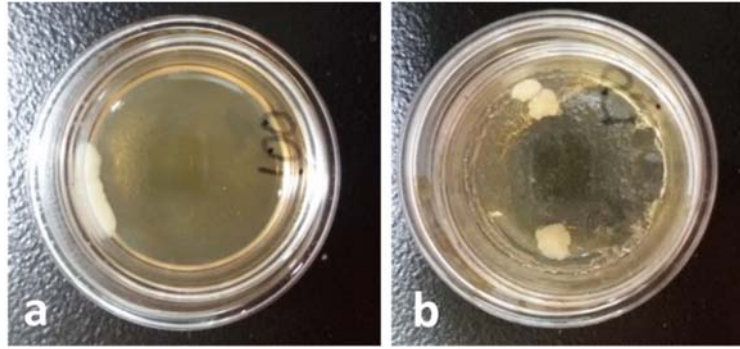


Figure S4. Cultivation of *Saccharomyces cerevisiae* (BY4741) on YPD media containing (a) 1.5 wt.% agar and (b) 1.0 wt.% peptide P1.

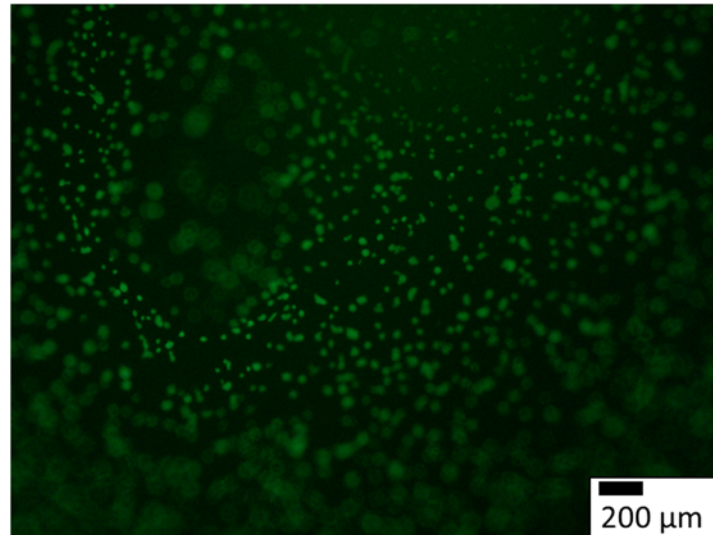


Figure S5. HeLa cells after 72 h incubation on 0.5 wt.% P1 hydrogel of DMEM medium without FBS. Living cells were dyed with ?? $\mu\text{g/ml}$ calcein AM and dead cells were dyed with ?? $\mu\text{g/ml}$ Hoechst 33342.

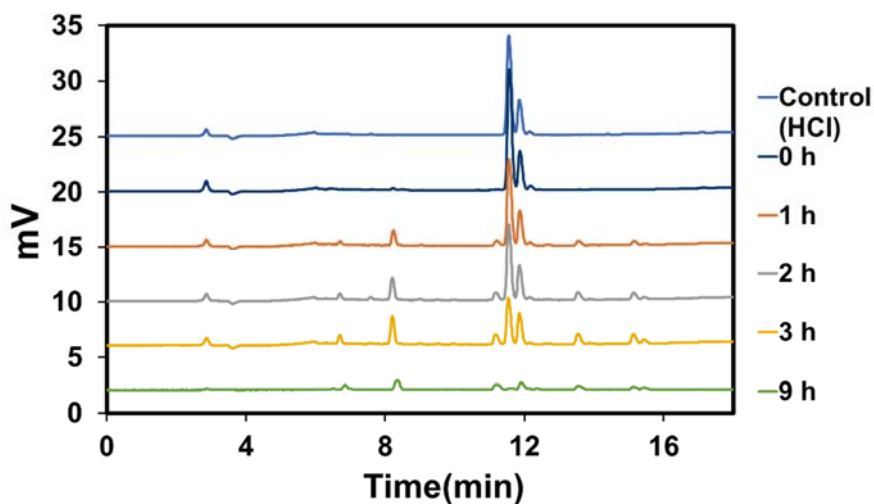


Figure S6. HPLC charts of enzymatic hydrolysis of P1 hydrogel.

MALDI-TOF/MS

1. Peptide P1 (Ac-Phe-Phe-Phe-Gly-Lys-OH)

MALDI-TOF/MS using α -cyano-4-hydroxycinnamic acid (CHCA) as matrix: $[M+H]^+$ calcd for $C_{37}H_{46}N_6O_7$, 686.3; found, 687.5.

2. Peptide P2 (Ac-Phe-Phe-Phe-Val-Lys-OH)

MALDI-TOF/MS using α -cyano-4-hydroxycinnamic acid (CHCA) as matrix: $[M+H]^+$ calcd for $C_{40}H_{52}N_6O_7$, 728.4; found, 728.3.

3. Peptide P3 (Ac-Phe-Phe-Phe-Ala-Lys-OH)

MALDI-TOF/MS using α -cyano-4-hydroxycinnamic acid (CHCA) as matrix: $[M+H]^+$ calcd for $C_{38}H_{48}N_6O_7$, 700.4; found, 701.4.

4. Peptide P4 (Ac-Phe-Phe-Phe-Lys-OH)

MALDI-TOF/MS using α -cyano-4-hydroxycinnamic acid (CHCA) as matrix: $[M+H]^+$ calcd for $C_{35}H_{43}N_5O_6$, 629.3; found, 629.8.

5. Peptide P5 (Ac-Phe-Phe-Phe-Gly-Lys-NH₂)

MALDI-TOF/MS using α -cyano-4-hydroxycinnamic acid (CHCA) as matrix: [M+H]⁺ calcd for C₃₇H₄₇N₇O₆, 685.3; found, 685.8.

6. Peptide P6 (H-Phe-Phe-Phe-Gly-Lys-NH₂)

MALDI-TOF/MS using α -cyano-4-hydroxycinnamic acid (CHCA) as matrix: [M+H]⁺ calcd for C₃₅H₄₅N₇O₅, 643.3; found, 643.7.

7. Peptide P7 (H-Phe-Phe-Phe-Gly-Lys-OH)

MALDI-TOF/MS using α -cyano-4-hydroxycinnamic acid (CHCA) as matrix: [M+H]⁺ calcd for C₃₅H₄₄N₆O₆, 644.3; found, 644.7.

8. Peptide P8 (Ac-Phe-Phe-Gly-Lys-OH)

MALDI-TOF/MS using α -cyano-4-hydroxycinnamic acid (CHCA) as matrix: [M+H]⁺ calcd for C₂₈H₃₇N₅O₆, 539.3; found, 539.5.

9. Peptide P9 (Ac-Phe-Phe-Val-Lys-OH)

MALDI-TOF/MS using α -cyano-4-hydroxycinnamic acid (CHCA) as matrix: [M+H]⁺ calcd for C₃₁H₄₃N₅O₆, 581.3; found, 581.9.

10. Peptide P9 (Ac-Phe-Phe-Ala-Lys-OH)

MALDI-TOF/MS using α -cyano-4-hydroxycinnamic acid (CHCA) as matrix: [M+H]⁺ calcd for C₂₉H₃₉N₅O₆, 553.3; found, 556.7.