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Hydrogel Formation by Short D-peptide for Cell-culture Scaffolds

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Abstract

The present study reports that a short oligopeptide D-P1, consisting of only five D-amino acids, self-assembled into entangled nanofibers to form a hydrogel that functioned as a scaffold for cell cultures. D-P1 (Ac-D-Phe-D-Phe-D-Phe-Gly-D-Lys) gelated aqueous buffer solution and water at a minimum gelation concentration of 0.5 wt. %. The circular dichroism (CD) measurements demonstrated the formation of a β-sheet structure in the self-assembly of D-P1. We investigated the gelation properties and CD spectra of both the D- and L-forms of the oligopeptide, and found only a minimal difference between them. The D-P1 hydrogel was resistant to a protease, whereas the L-P1 hydrogel was rapidly degraded. Both oligopeptides exhibited nontoxic properties to human cancer cells and embryoid bodies (EBs) derived from human-induced pluripotent stem cells. Additionally, we succeeded in forming spheroids of HeLa cells on the D-P1 hydrogel, which indicates the potential of this hydrogel for 3-dimensional cell culture.

Keywords: cell scaffolds, cytotoxicity, D-amino acids, induced pluripotent stem cells (iPSCs), protease, short D-oligopeptide.

1. Introduction

Amino acids are versatile building blocks for creating functional assemblies. There are a number of reports on low molecular weight gelators (LMWGs) based on amino acids and peptide [1-4]. The use of amino acids provides biodegradability and biocompatibility [5]. An LMWG, in general, forms a physical gel after preparation of an isotropic solution. Gelator molecules self-assemble based on multiple non-covalent interactions (hydrogen bonding, van der Waals interaction, π-π stacking among others), resulting in fibrous assemblies that form a three-dimensional (3D) network structure. The 3D network traps solvent to form a gel [6,7]. A number of studies on LMWGs proposed a broad range of applications, including as catalysts for organic reactions, pollutant removal, nanofabrication

template, wound-healing scaffold, biomolecule encapsulation, tissue engineering and emulsion stabilizer [8-17].

α-Amino acids (except for Gly) have chirality, and this chirality plays an important role in nature. Most proteins contain only L-amino acids, because the ribosome selectively incorporates L-amino acids to produce polypeptides [18-22]. Proteolytic enzymes recognize peptide bonds between L-amino acid residues. The use of D-amino acids produces peptides that are resistant to hydrolysis by proteolytic enzymes [22-24]. In the last decade, there have been a considerable number of studies on peptides composed of D-amino acids in various fields, including biotechnology, medicine and tissue engineering among others [25-33]. For example, Marchesan *et al.* synthesized tripeptides composed of mixtures of D- and L-amino acids, ^{D}VFF and ^{D}FFV. They revealed that the mixture of D- and L-amino acids in tripeptides drastically promoted their self-assembly to produce hydrogels at physiological pH and demonstrated the importance of the position of different chirality in the tripeptides [34,35]. The hydrogel of the tripeptide was available for mouse cell culture. Xu *et al.* conjugated D-amino acid and glycoside to produce an LMWG having proteolytic resistance and demonstrated the high stability of D-peptides within the mouse body [36]. This group also reported functional self-assembly of D-peptide derivatives, which improved selectivity in enzyme inhibition to function as a non-steroid anti-inflammatory drug. Nanofibrils of D-peptide derivatives also induced apoptosis of cancer cells [37-39]. Ou *et al.* prepared a long peptide, DFEFKDFEFKYRGD, to produce a hydrogel in which 3D cell culture of HeLa cells was achieved [40].

In our previous study, we developed short oligopeptides, composed of only five amino acids, which assembled into entangled nanofibers to form hydrogels at low concentrations (< 1.0 wt. %) [41]. In particular, the peptide L-P1 (Ac-Phe-Phe-Phe-Gly-Lys) displayed high gelation ability, in which the minimum gelation concentration (MGC) was 0.5 wt. %. While

it showed low cytotoxicity, the L-P1 hydrogel was readily decomposed by a proteolytic enzyme, indicating its low potential for cell scaffolds. In the present study, we aimed to produce a hydrogel of a short D-oligopeptide that can be used as a cell scaffold. We employed D-amino acids to prepare D-P1 (Ac-D-Phe-D-Phe-D-Phe-Gly-D-Lys). The gelation properties of D-P1 and its resistance to a proteolytic enzyme were investigated. Finally, we succeeded in cell culturing and the formation of a cellular spheroid on the D-P1 hydrogel.

2. Experimental

2.1. Materials

Amino acid-preloaded Alko resin, Fmoc-protected amino acids, O-(1H-benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxy-1H-benzotriazole hydrate (HOBt·H2O) and triisopropylsilane (TIPS) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). N,N-Diisopropylethylamine (DIEA) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Kaiser reagents for ninhydrin tests were purchased from Kokusan Chemical (Tokyo, Japan). Cell counting kit-8 and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) were purchased from Dojindo Molecular Laboratories, Inc. (Kumamoto, Japan). Fetal bovine serum (FBS) was obtained from Sigma Aldrich. Inc. (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin and 2.5g/L trypsin solution containing 1 mM ethylenediaminetetraacetic acid (EDTA) were purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Water used was produced by a Millipore water purification system (Elix UV 3 system, Millipore, Molsheim, France). All chemicals were used without further purification.

2.2. Synthesis of peptides

Peptides were synthesized on Alko resin using the standard Fmoc solid-phase peptide synthesis method. An Fmoc-protected amino acid (3 equivalents) was coupled to 0.3 mmol amino-acid-preloaded Alko resin, using HBTU and HOBt·H2O as the coupling agents in the presence of DIEA in dimethylformamide (DMF). Qualitative ninhydrin tests were used to confirm the completion of each coupling reaction. The crude peptides were cleaved manually from the resin and the side chains were deprotected using a trifluoroacetic acid (TFA)/TIPS/water mixture (95: 2.5: 2.5 in volume) at room temperature for 2 h. The crude peptides were precipitated and washed three times with diethyl ether and centrifuged at 7000 rpm for 5 min. The precipitates were lyophilized overnight. The high-performance liquid chromatography (HPLC) system, Shimadzu LC-20AT equipped with a UV-vis detector SPD-20A (Shimadzu corp., Tokyo, Japan), was used to purify all the 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 purified peptides were obtained after lyophilization of the collected HPLC fractions. The final compound was characterized by matrix-assisted laser desorption ionization−time of flight mass spectrometry (MALDI-TOF/MS) using an UltrafleXtreme™ mass spectrometer (Bruker, Billerica, MA) (MS data were in Supplementary data). The obtained peptides were stored at −4 °C until use.

2.3. Preparation of peptide hydrogels

Pentapeptide was placed in a glass tube (diameter 8 mm) and dissolved in an aqueous phosphate buffer solution (pH 7.4, 50 mM) by heating on a sand bath at 85 °C followed by ultrasonication for 5 min. Heating at 85 °C was to ensure all the gelators dissolved in aqueous solutions. The solutions were cooled slowly at room temperature to form a hydrogel. Gelation was confirmed by inverting the glass tube containing the solution.

2.4. Transmission electron microscopy (TEM) observation

Carbon-coated copper grids (ELS-C10, Okenshoji Co., Ltd., Tokyo, Japan) were used for TEM observation. A drop of hydrogel sample was placed on a grid and stained with an aqueous solution containing potassium phosphotungstate (2.0 wt. %). Sample-loaded grids were vacuum-dried and observed using a JEOL JEM-2100 F transmission electron microscope (Tokyo, Japan) at an operating voltage of 200 kV.

2.5. Rheology measurements

Rheology measurements were performed 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. Peptide hydrogels at their MGCs were loaded on a sample plate whose temperature was set at 90 °C. The sample plate was gradually cooled to 25 °C (over 30 min) and the measurement was started.

2.6. Circular dichroism (CD) spectroscopy analysis

The secondary structures of the peptides in solution were analyzed using a 0.1-cm-thick quartz cell on a Jasco J-725K spectropolarimeter (Jasco corp., Tokyo, Japan) with four accumulations, 4 s integrations, a step size of 1.0 nm and a bandwidth of 1.0 nm over a range of wavelengths from 200 to 250 nm. A peptide solution was prepared at various concentrations in a 25 mM phosphate buffer solution and was left for 1 h. Spectra were recorded with increasing temperature from 25 to 90 °C.

2.7. Hydrogel decomposition using a proteolytic enzyme

α-Chymotrypsin was used for peptide hydrolysis. A stock solution (0.02 wt. % chymotrypsin in 20 mM HCl aqueous solution) was prepared. A hydrogel (250 μL) was prepared using a phosphate buffer solution (pH 7.4, 0.1 M) containing 1.0 wt. % peptide in a glass vial and the enzyme stock solution (25 μL) was added, followed by incubation at 40 °C. Fresh phosphate buffer solution (250 μL, pH 7.4, 0.1 M) was added to the glass vial and shaken vigorously to dissolve the hydrogel. Aliquots (100 μL) were periodically taken and applied to HPLC analysis. As a control, 20 mM HCl aqueous solution (25 μL) was added to the hydrogel instead of the enzyme stock solution.

2.8. Cytotoxicity study and live-dead assays of HeLa cells

HeLa cells were cultured in a DMEM solution supplemented with 10 % (v/v) FBS and 1 % (v/v) penicillin-streptomycin using a plate dish (diameter 100 mm) under 5 % CO_2 atmosphere at 37 °C. The culture medium was exchanged every 72 h with the fresh medium, and the cells were harvested with phosphate-buffered saline (PBS) containing 0.25 g/L trypsin 0.1 mmol/L EDTA and counted using a cell counter (Cell Counter model R1, Olympus, Tokyo, Japan).

The resulting cell suspension was diluted in DMEM medium and the cells were seeded at a density of 5,000 cells per well in a 96-well microplate (Iwaki 3860-096, Asahi Glass Co., Ltd., Tokyo, Japan) and cultured for 24 h. The culture medium was exchanged with 100 μL DMEM medium containing peptide at a given concentration. After the cells were cultured for 24 h, cell viability was determined using Cell counting kit-8 and measured by the microplate reader (SH9000, Hitachi igh-Technologies Corporation, Tokyo, Japan).

To observe the living and dead HeLa cells on a hydrogel, a cell suspension (100 μL) containing 5,000 cells was placed on a hydrogel of 0.5 wt. % peptide in a well of a 96-well microplate. After 3 days, 100 μL PBS solution containing 2.7×10^{-4} μg/μL calcein AM and 3.4×10^{-2} μg/μL Hoechst was added to the well for cell staining. Thirty minutes after the

addition of the dyes, living and dead cells (green and blue, respectively) were observed using a fluorescence microscope (Olympus IX71 equipped with cellSens).

2.9. Cytotoxicity study and live-dead assays of embryoid bodies (EBs)

Induced pluripotent stem cells (iPSCs) were cultured in StemFit AK02N medium (Ajinomoto Co., Tokyo, Japan) under 5 % CO_2 atmosphere at 37 °C and the culture plates were pre-coated with recombinant laminin-511 E8 fragments (0.5 mg/cm2) (iMatrix-511, Nippi, Tokyo, Japan). The medium was changed every 2 days and the cells were passaged every 7 days using 0.5 × TrypLE Select (1 × TrypLE Select diluted 1:1 with 0.5 mM EDTA/PBS (−)) (Life technologies, Thermo Fisher Scientific Inc., Tokyo, Japan) and Rock inhibitor (Y-27632, Wako Pure Chemical Industries).

The resulting cell suspension was diluted in Primate ES cell medium (Reprocell, Kanagawa, Japan) and Y-27632 (20 mM) at a cell density of 5×10^5 cells per well (100 μL) in a 96-well microplate (Prime Surface MS-9096M, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and cultured for 2 days. Subsequently, the embryoid bodies (EBs) were ready to be cultured on the hydrogel.

To observe the living and dead cells of EBs on a hydrogel, an EBs suspension (100 μL) was placed on a hydrogel of 0.5 wt. % peptide in a well of a 96-well microplate. The medium was changed every two days. After 4 and 8 days, 100 μL PBS solution containing 0.17 g/μL calcein AM and 0.25 μg/μL propidium iodide (PI) was added to the well for cell staining. Thirty minutes after adding the dyes, living and dead cells (green and red, respectively) were observed using a fluorescence microscope (Olympus IX71 equipped with cellSens).

2.10. 3D cell culture of HeLa cells

A 100 μL hydrogel of 1.0 wt. % D-peptide was prepared using DMEM medium containing 10 % (v/v) FBS and 1 % (v/v) penicillin-streptomycin in a well of a 96-well microplate (Prime Surface 96U, Sumitomo Bakelite Co., Ltd.). One day after the hydrogel preparation, a HeLa cell suspension (10,000 cells in 100 μL DMEM solution) was placed on the hydrogel and the cells were cultured under a 5 % CO_2 atmosphere at 37 °C. The formation of the spheroid of cells on the hydrogel was observed using a microscope (Olympus IX71).

3. Results and discussion

3.1. Peptide design and self-assembly

Several groups reported D-peptide-based hydrogelators that were stable in the presence of proteolytic enzymes [26-28]. In our previous study, we found that an N-acetylated L-pentapeptide, Ac-Phe-Phe-Phe-Gly-Lys-OH (termed L-P1) functioned as a good hydrogelator for various kinds of aqueous solutions and displayed low cytotoxicity [40]. Here we employed D-amino acids to synthesize an N-acetylated D-pentapeptide, Ac-D-Phe-D-Phe-D-Phe-Gly-D-Lys-OH (termed D-P1, Fig. 1A) and evaluated its gelation ability. D-P1 had the identical amino-acid sequence as L-P1. Table 1 summarizes the gelation ability of D-P1 and L-P1, and these peptides displayed the same minimum gelation concentration for aqueous buffers (phosphate, HEPES and Tris buffers) and water. These results were in agreement with the studies of Zhang *et al.* and Liu *et al.*, in which the chirality of peptides did not affect the ability of the molecular self-assembly, and the L- and D-peptides formed hydrogels in a similar manner [26-28].

The gelation of aqueous solutions by D-P1 produced an appearance of transparent hydrogels for the buffer solutions tested (Fig. 1B).

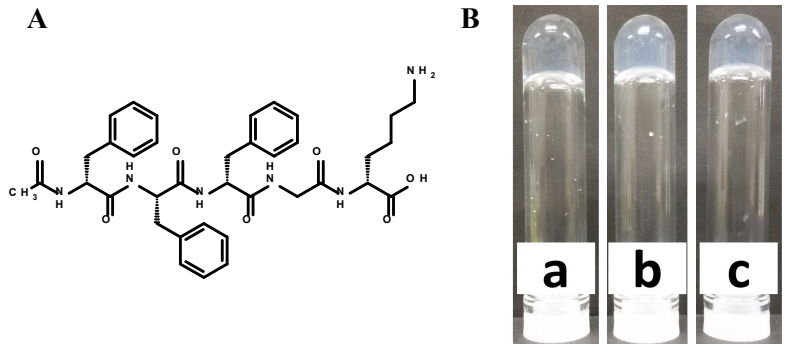


Fig. 1 A) Chemical structures of D-P1 peptide (Ac-D-Phe-D-Phe-D-Phe-Gly-D-Lys-OH). B) Hydrogels prepared with peptides D-P1 at their minimum gelation concentrations in buffer solutions (50 mM and pH 7.4). (a) Phosphate buffer, (b) Tris-HCl buffer and (c) HEPES buffer.

Table 1. Gelation properties of the synthesized oligopeptides.

Peptide	Amino acid sequences	Gelation test in			
		Phosphate Buffer	Tris-HCl Buffer	HEPES Buffer	Water
L-P1*	Ac-Phe-Phe-Phe-Gly-Lys-OH**	G (0.5) ***	G (0.5)	G (0.5)	G (0.5)
D-P1	Ac-D-Phe-D-Phe-D-Phe-Gly-D-Lys-OH	G (0.5)	G (0.5)	G (0.5)	G (0.5)

* The results of L-P1 are from ref. [41]. **Ac represents an acetyl group. **G indicates gel. Minimum gelation concentrations in wt. % are given in parentheses. All buffers are 50 mM and pH 7.4

3.2. TEM observation

TEM observation was performed for the hydrogels prepared with the D-P1 and L-P1 to obtain morphological information (Fig. 2). The molecules of peptide gelator self-assembled through noncovalent interactions such as hydrophobic interaction, π–π interactions and hydrogen bonding. Both the hydrogels exhibited nanofibrous networks similar to other LMWGs reported previously. The entangled (or branched) fibrous networks had a fiber width of approximately 30–60 nm and were several micrometers in length. These nanofibrous structures were composed of self-assemblies of the D-type peptide in an aqueous solution. The only difference between the peptides was their chirality.

Therefore, it appears that the chirality of the peptides did not significantly affect the resultant morphology of their self-assembly [23,26].

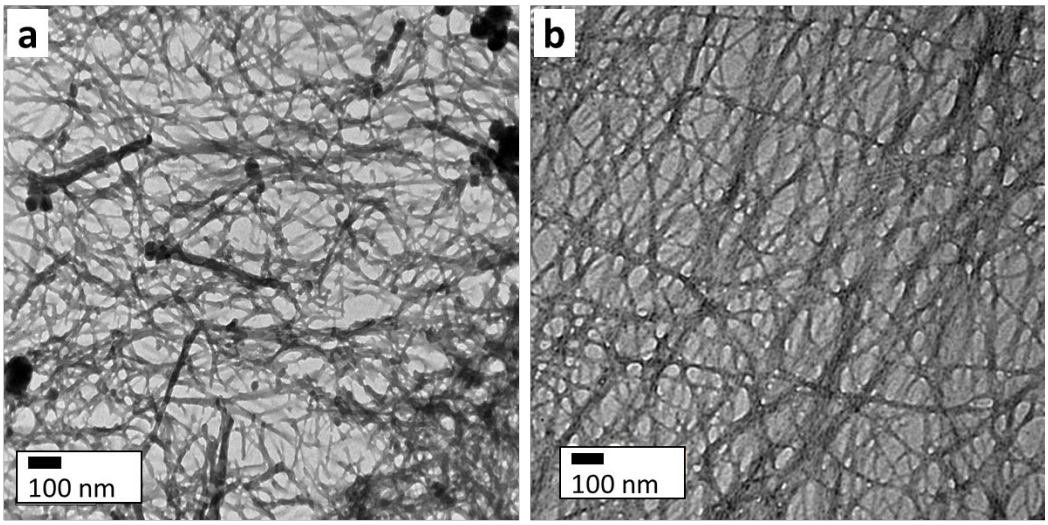


Fig. 2 Transmission electron microscopy images of hydrogels with (a) D-P1 and (b) L-P1 in 50 mM phosphate buffer solution (pH 7.4) at their minimum gelation concentrations (0.5 wt%). Panel B was reproduced from ref 41.

3.3. Rheological measurements

Rheological experiments were performed for hydrogels prepared with D-P1 and L-P1. The storage modulus (G') and the loss modulus (G'') were measured in the frequency sweep with a fixed strain of 0.1 % (Fig. 3). Both hydrogels displayed a higher G' than G'', which is an indication of the dominant elastic behavior of a gel [42-45]. The D-P1 hydrogel exhibited a G' approximately eight-fold higher than G'' and L-P1 hydrogel showed G' seven-fold higher than G'' over the tested range, which indicated the formation of a stable gel. The stable gel would be associated with the 3D fibrous networks composed of peptide assemblies. D-P1 and L-P1 hydrogels showed similar rheological profiles.

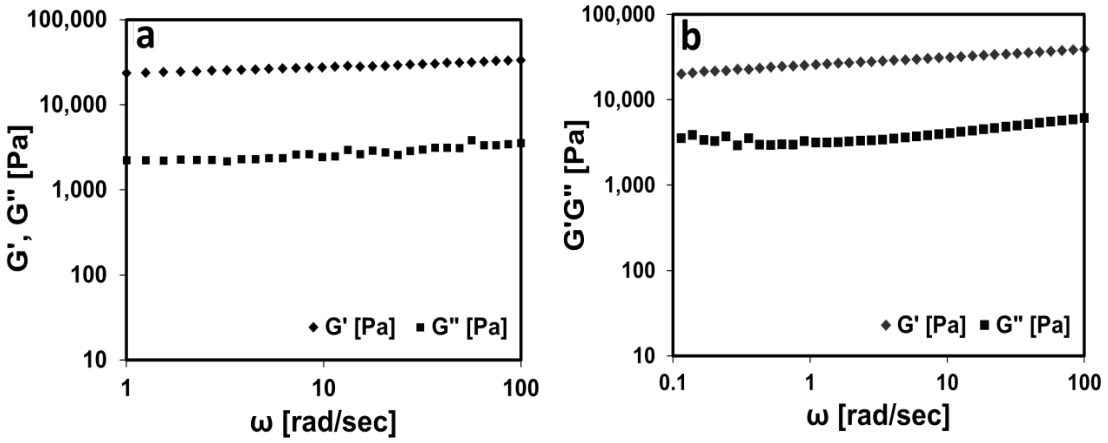


Figure 3. Rheological properties of (a) D-P1 and (b) L-P1 hydrogels in 50 mM phosphate buffer solution (pH 7.4) at their minimum gelation concentrations. Panel b was reproduced from ref 41.

3.4. Characterization of secondary structure

CD measurements were performed to obtain information on the secondary structures of the self-assemblies of D-P1 and L-P1. The L-P1 hydrogel had a large negative band at approximately 225 nm (Fig. 4a). D-P1 had a mirror-image CD spectrum to that of L-P1. Both the results indicate the presence of β-sheet-like structures in their gel phases, which were in agreement with the results of previous reports [30-33].

Low concentrations of peptide D-P1 (0.1 wt. % – 0.3 wt. %) resulted in negative bands at approximately 220 nm (Fig. 4b), meaning no formation of a β-sheet-like structure. These results imply that the peptide concentration was critical for the self-assembly of peptide P1. Fig. 4c shows the effect of temperature on the CD spectra of peptide D-P1 (0.4 wt. %). The large positive band at approximately 225 nm decreased with an increase of temperature. When the temperature increased to 70 and 90 °C, the positive band at approximately 225 nm disappeared and a negative band appeared at approximately 223 nm. These results indicate the decrease of a β-sheet-like structure and conversion to random coils as the temperature increased, which would lead to the thermoreversible gel-sol transition. Indeed, the naked-eye

observation demonstrated that the 0.5 wt. % D-P1 hydrogel displayed a reversible gel-sol transition at approximately 72 °C, which was consistent with that of L-P1 [41].

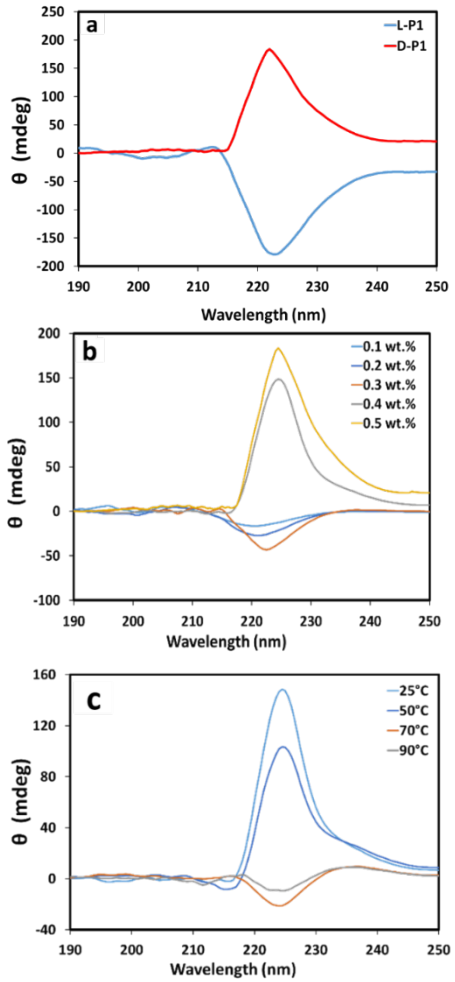


Fig. 4 (a) Circular dichroism (CD) spectra of a 25 mM phosphate buffer solution containing D-P1 or L-P1 hydrogel at 25 °C. Peptide concentrations were 0.5 wt. %. CD spectra of a 25 mM phosphate buffer solution containing peptide D-P1 of (b) different concentrations at 25 °C and (c) at various temperatures (0.4 wt. %).

3.5. Resistance to a proteolytic enzyme

Because D-peptides are in general resistant to hydrolysis by proteolytic enzymes, we evaluated the enzymatic degradation of the hydrogels. α-Chymotrypsin was used as a proteolytic enzyme that preferentially cleaves peptide bonds where the carboxyl side of an

amide bond is a large aromatic amino acid (tyrosine, tryptophan and phenylalanine) in an L-amino acid residue. No degradation occurred of the D-P1 hydrogel even after 7 days in the presence of α-chymotrypsin (Fig. S2). This result was in accordance with the results by Xu *et al.*, in which the use of D-amino acids increased the stabilities of their peptide derivatives towards a proteolytic enzyme [36,46]. This result is also important in 3D culturing of the cells since the cell proliferation proceeds over a couple of days.

3.6. Cytotoxicity study and live-dead assays of HeLa cells

Some D-amino acids, including D-Ala and D-Asp, were found at remarkably high concentrations in pathological sites, which indicates some pathological activities of D-amino acids [47]. There are also reports of physiological activities of D-amino acid-based peptides [48-50]. Here, we investigated the cytotoxicity of D-P1 and L-P1 on human cancer cells (HeLa cells). After 24 h of incubation with D-P1 or L-P1 (0.05 wt. % – 0.4 wt. %), the cell viability with both peptides was maintained at > 60 % (Fig. 5). Our previous LMWG composed of L-amino acids displayed high cytotoxicity for HeLa cells only at 0.02 wt. % [51]. These results indicate that D-P1 and L-P1 had low toxicity toward HeLa cells. The lowest concentration of D-P1 peptide (0.05 wt.%) exhibited > 100% cell viability, which meant the presence of D-P1 peptide did not affect the cell viability and it kept cell proliferation. There are various options for a cell-culture medium. DMEM used in the present study contains nutrients at higher concentrations than Eagle's minimal essential medium, which might also lead to the high cell viability in the presence of the peptides.

The previous observation was done in order to confirm the low-cytotoxicity of D-P1 peptide. To directly observe the cell living on the hydrogels of the peptides, the live/dead assay for HeLa cells using a fluorescent dye was conducted. HeLa cells were seeded on both

D-P1 and L-P1 hydrogels and cultured with DMEM solution in the absence of FBS. Green fluorescence indicated that the cells remained alive on both hydrogels even after a 72 h of incubation (Fig. 6). These results agreed with those of the above cytotoxicity tests.

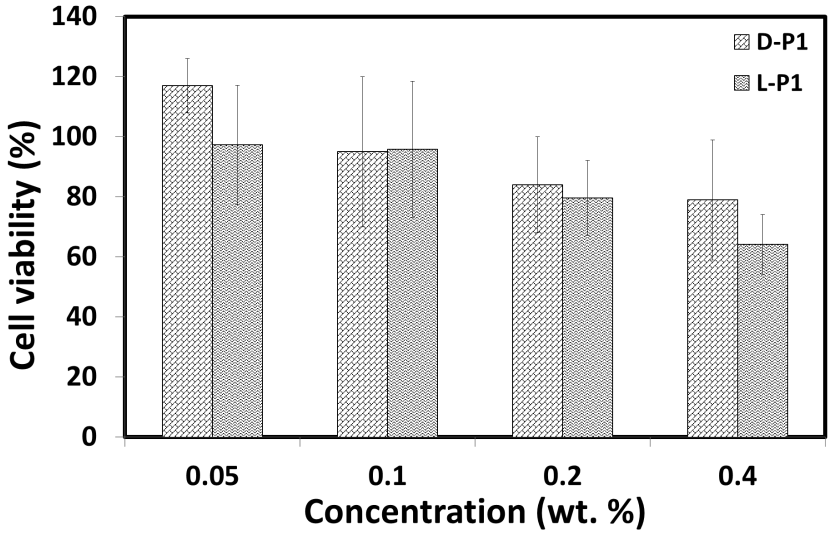


Fig. 5 Cell viability of HeLa cells after 24 h of incubation with peptides D-P1 and L-P1. The results of L-P1 are from ref. [41].

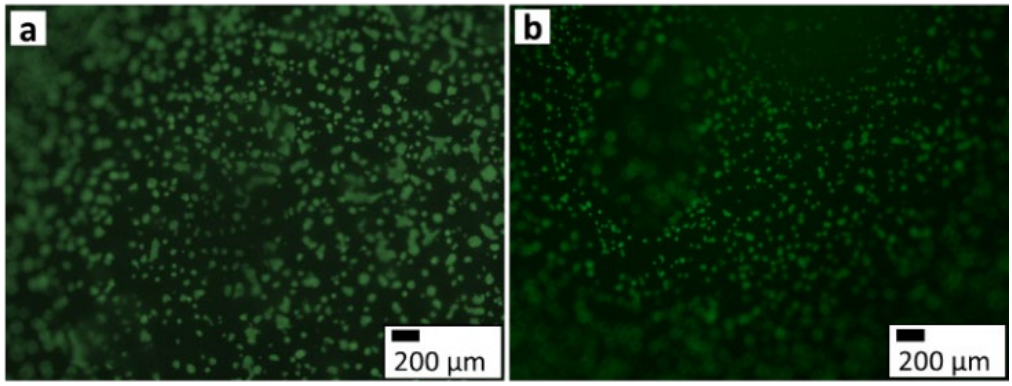


Fig. 6 HeLa cells after 72 h of incubation on (a) D-P1 hydrogel and (b) L-P1 hydrogel (without FBS). Living cells were stained with 2.7×10^{-4} µg/µL calcein AM.

3.7. Spheroid formation

3D cell culture is one of the interesting areas of the medical and biomaterial fields since it mimics the microenvironment of living tissue [16,52]. A spheroid of cells is one of the simple forms of 3D cell culture. We attempted the spheroid formation of HeLa cells on a D-P1 hydrogel. A hydrogel (100 μL) prepared with DMEM medium and D-P1 was placed in a 96-microplate well and the equivalent volume of a cell suspension (in DMEM medium) was poured in gently. With time, HeLa cells proliferated and successfully formed spheroids on the hydrogel (Fig. S3). These results indicated that the cells interacted with the hydrogel and showed the stability of D-P1 hydrogel in the presence of living cells. By contrast, the culture of HeLa cells on an L-P1 hydrogel resulted in the decomposition of the hydrogel, because proteolytic enzymes in FBS degraded the L-P1 peptide [41].

3.8. Cytotoxicity study and live-dead assays of embryoid bodies (EBs)

EBs are aggregates of pluripotent cells that are induced to differentiate by changes in the culture medium (e.g., removing the factors that support pluripotency) to allow the cells to interact in 3D cell aggregates [53-55]. The differentiation of EBs is a common pathway to produce specific cell lineages from pluripotent cells. Here, we studied the cytotoxicity of D-P1 and L-P1 hydrogel towards EBs. The live/dead assay of EBs was conducted using fluorescent dyes (calcein AM for living cells and PI for dead cells). EBs were seeded on D-P1 and L-P1 hydrogels and cultured. Fluorescent microscopy images showed green fluorescence on the EBs in all cases at days 8, indicating that the EBs remained alive on both hydrogels even after an 8-day incubation (Fig. 7). Red fluorescence indicating the co-existence of dead cells in the EBs was in similar intensity to those without hydrogel at days 8. Based on the observation by the naked eyes, their size and shape were comparable to those of the control (without a hydrogel). These results indicate that D-P1 and L-P1 had low toxicity toward the EBs, which were non-cancer cells, and also that the gels did not affect negatively to the growth of the EBs. These results indicated that the D-P1 and L-P1 hydrogels can provide a platform for the growth of

EBs since they did not give any negative effect on the growth of the EBs. Ramón-Azcón *et al.* and Nieden *et al.* reported polymer hydrogels as cell scaffolds for EBs [56, 57]. The present study revealed that low-molecular-weight hydrogels can also function as cell scaffolds for EBs.

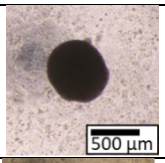
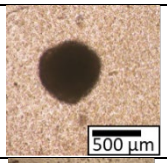
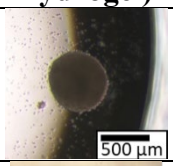
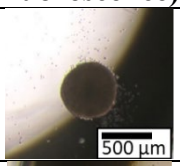
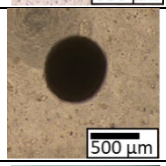
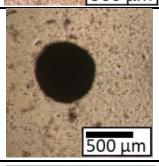
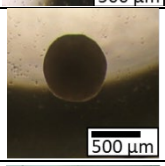
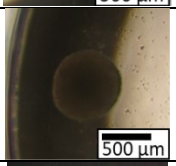
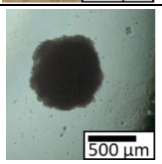
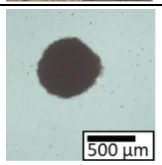
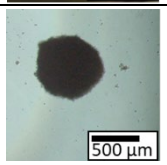
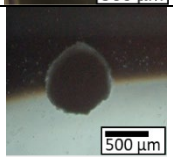
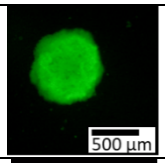
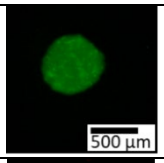
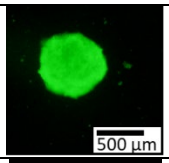
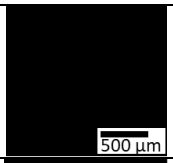
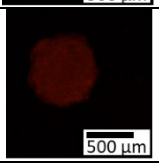
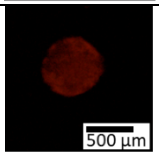
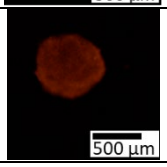
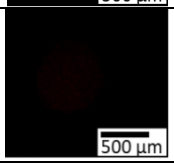
Observation		EBs on D-P1 hydrogel	EBs on L-P1 hydrogel	Positive Control (EBs without a hydrogel)	Negative Control (EBs without fluorescence)
Day 0 (Phase contrast)					
Day 2 (Phase contrast)					
Day 8	Phase contrast				
	Fluorescence image (calcein AM)				
	Fluorescence image (PI)				

Fig. 7 Microscopic observation of embryoid bodies (EBs) cultured on hydrogels and without a hydrogel.

4. Conclusion

In summary, we synthesized the D-form and the L-form of a short peptide, D-P1 and L-P1, respectively, and evaluated their gelation properties and cytotoxicity. Both gelated aqueous buffer solutions at physiological pH at the same low concentration (0.5 wt. %). The resultant hydrogel exhibited nanofibrous networks. Despite the similarity in the gelation properties, the D-P1 hydrogel was resistant to a proteolytic enzyme. Both peptides displayed low toxicity towards human cancer cells and spheroids of HeLa cells were successfully formed on the D-P1 hydrogel. The hydrogels did not affect the growth of the EBs, which were derived from

the iPSCs. These indicate the potential of a D-P1 hydrogel for 3D cell culture in the field of regenerative medicine.

Declaration of competing interest

There are no conflicts to declare.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found online version at https:// doi.org/10.1016/j.msec.xxxxxx.

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Supplementary information

Hydrogel Formation by Short D-peptide for Cell-culture Scaffolds

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Results

MALDI-TOF/MS

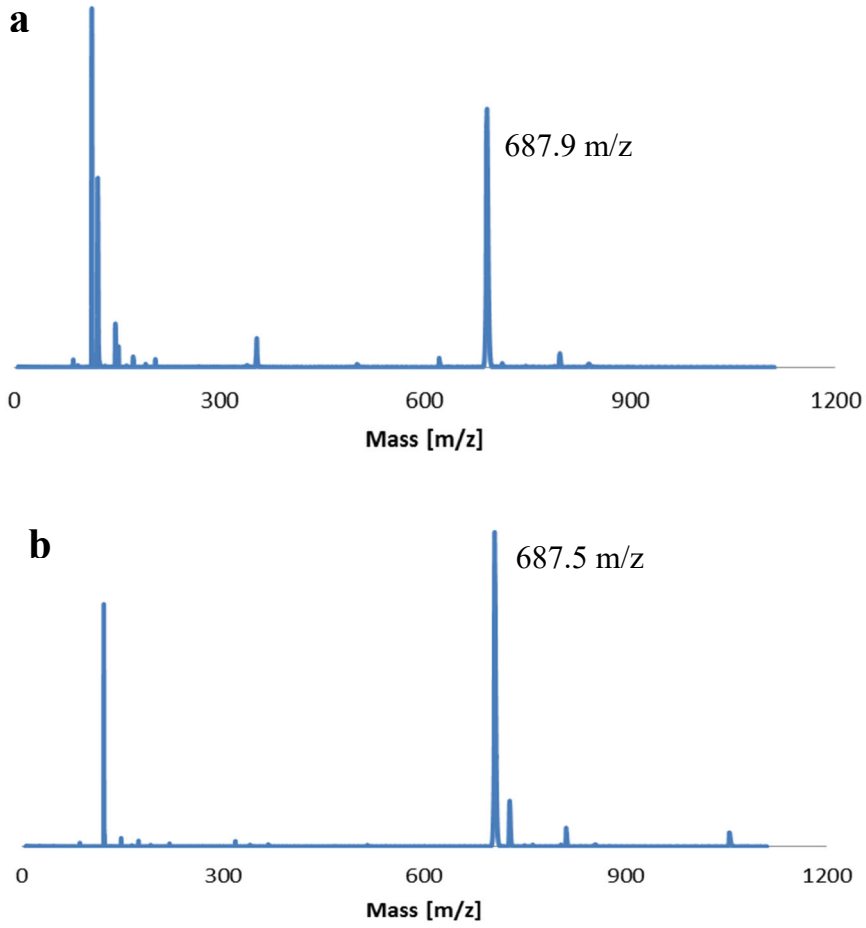

Figure S1. Molecular weight of peptide D-P1 (a) and L-P1 (b).

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

   MALDI-TOF/MS using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix: $[M+H]^+$ calculated for $C_{37}H_{46}N_6O_7$, 687.4; found, 687.9.

2. Peptide L-P1 (Ac-Phe-Phe-Phe-Gly-Lys-OH)

   MALDI-TOF/MS using CHCA as the matrix: $[M+H]^+$ calculated for $C_{37}H_{46}N_6O_7$, 687.4; found, 687.5.

Resistance to a proteolytic enzyme

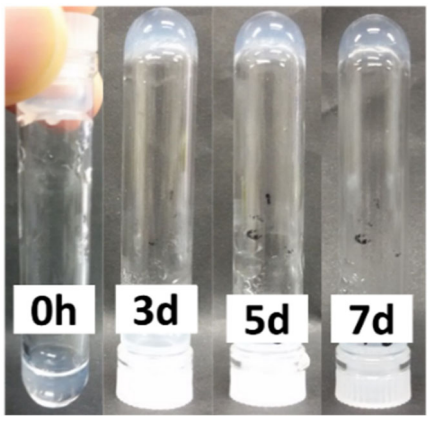

Fig. S2 Photos of α-Chymotrypsin resistance of D-P1 hydrogel.

Spheroid formation

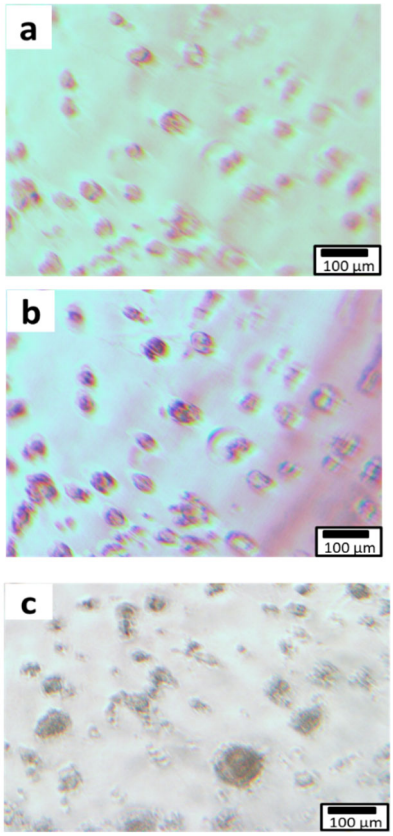

Fig. S3 Spheroid formation of HeLa cells on a D-P1 hydrogel after 1 d (a), 3 d (b) and 5 d (c) of incubation.