The mechanical stimulation of cells in 3D culture within a self-assembling peptide hydrogel

Yusuke Nagai [1,2], Hidenori Yokoi [2], Keiko Kaihara [1], and Keiji Naruse [1]

[1] Cardiovascular Physiology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, [2] Menicon Co., Ltd.

Abstract

The aim of this present study was to provide a scaffold as a tool for the investigation of the effect of mechanical stimulation on three-dimensionally cultured cells. For this purpose, we developed an artificial self-assembling peptide (SPG-178) hydrogel scaffold. The structural properties of the SPG-178 peptide were confirmed by attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and transmission electron microscopy (TEM). The mechanical properties of the SPG-178 hydrogel were studied using rheology measurements. The SPG-178 peptide was able to form a stable, transparent hydrogel in a neutral pH environment. In the SPG-178 hydrogel, mouse skeletal muscle cells proliferated successfully (increased by 12.4 ± 1.5 times during 8 days of incubation; mean ± SEM). When the scaffold was statically stretched, a rapid phosphorylation of ERK was observed (increased by 2.8 ± 0.2 times; mean ± SEM). These results demonstrated that the developed self-assembling peptide gel is non-cytotoxic and is a suitable tool for the investigation of the effect of mechanical stimulation on three-dimensional cell culture.

Keywords: Cell Proliferation; Self-assembly; Hydrogel; Scaffold; Mechanical Strain

1. Introduction

The goal of tissue engineering is to restore diseased or damaged tissue by delivering functional cells, scaffolds, and signal molecules such as growth factors to the affected area. The scaffolds should permit the release of the signal molecules and the ingress of nutrients and oxygen to keep the implanted cells alive. Furthermore, to elicit the implanted cell functions, the scaffolds should transmit several mechanical stimulations. The effects of mechanical stimulation have been proven in several cell culture systems, e.g., cardiomyocytes [1, 2], chondrocytes [3, 4], and others [5, 6, 7]. Animal-derived materials such as collagen and Engelbreth-Holm-Swarm (EHS) gels are widely used as scaffolds for tissue engineering because of their general compatibility with living tissues [8]. However, those scaffolds often contain growth factors, which may interfere with the estimation of the effects of the mechanical stimulation [9, 10]. Furthermore, such animal-derived materials can cause allergic reactions [11, 12] and carry dangerous pathogens including prions that cause a variety of neurodegenerative diseases in humans and animals. Evidence for the transmission of bovine spongiform encephalopathy prions to humans has been reported [13]. Other viruses might also be carried as pathogens in animal-derived scaffolds. Thus, there is a need for alternative sources of animal-derived scaffolds.

Self-assembling peptides are one of the candidate materials to solve these problems. The complete sequence of a self-assembling peptide was originally found in a region of alternating hydrophobic and hydrophilic residues in zuotin [14], which is characterized by a stable β-sheet structure that undergoes self-assembly into nanofibers. The nanofibers form interwoven matrices that further form a hydrogel scaffold [15, 16]. These hydrogel systems are well characterized and have already been employed in a variety of tissue engineering studies [17-20], drug delivery systems [21, 22], and

hemostatic applications [23]. Self-assembling peptides are a 100 % chemically synthesized material. Therefore, the use of self-assembling peptide hydrogels can minimize the risk of biological contamination and the influence from the undefined factors in EHS gels.

However, a typical self-assembling peptide gel, RADA16 (RADARADARADARADA; R=arginine, A=alanine, and D=aspartic acid) gel, has a very low pH (approximately 3-4), thereby retaining the potential to harm inner cells and host tissues. In one particular case, a gel required one week to gradually change its pH from acidic to neutral by a solvent substitution [24]. In addition, an important drawback is that the RADA16 hydrogel structure is unstable under neutral conditions. After the neutralization procedure, the hydrogel tends to break under mechanical stress [25]. Furthermore, once the hydrogel is stirred, the electrostatic interactions between the protonated arginine (+) and the deprotonated aspartic acid (-) within the RADA16 peptide strongly occur and yield a precipitate [26].

The aim of this study was to provide a scaffold as a tool for the investigation of the effect of mechanical stimulation on three-dimensionally cultured cells without any interference from undefined factors such as the growth factors in animal-derived scaffolds. For this purpose, we developed a self-assembling peptide, SPG-178 (Self-assembling Peptide Gel, amino acid sequence #178; [CH_3CONH]-RLDLRLALRLDLR-[$CONH_2$]; R=arginine, L=leucine, D=aspartic acid, and A=alanine; Fig. 1). It is well known that a protein reaches its minimum solubility at its isoelectric point, where the protein has a zero net charge. This property of proteins is closely related to the instability of the hydrogel formed by the RADA16 peptide, whose isoelectric point is 6.1 [26]. Therefore, the isoelectric point of the SPG-178 peptide was designed to be 11.5 by employing four cationic arginine and two anionic aspartic acid

residues. Furthermore, leucine residues were employed to increase the hydrophobic interaction among the SPG-178 peptides, which was the main driving force of the self-assembly, and stabilize the hydrogel formation.

We report here on the biocompatibility of a developed self-assembling peptide hydrogel and its ability to transmit mechanical stimulation. Murine C2C12 myoblast cells were used for all of the cell culture experiments because they are known to be acutely responsive to mechanical stimulation.

2. Materials and methods

2.1. Self-assembling peptide SPG-178

The self-assembling peptide SPG-178, [CH_3CONH]-RLDLRLALRLDLR-[$CONH_2$], was synthesized by a solid-phase method using standard Fmoc strategy (see Supplementary Data and Fig. S1). The peptide powder was dissolved in a 10 % (w/v) sucrose solution. Then the peptide solution was sterilized by filtration through a 0.22 μm filter. The pH of the peptide solution was adjusted to approximately pH=6.5 by adding aliquots of a 0.5 % (w/v) sodium hydrogen carbonate solution. The final concentration of the peptide in the solution was 2.4 mM (0.4 % w/v).

2.2. CPK model of SPG-178 peptide

The molecular models of the anti-parallel β-sheet structure and the fiber formation of the SPG-178 peptide were produced using Facio, a 3D-graphics program, and employing Tinker with a charm 22 force field parameter

(http://www1.bbiq.jp/zzzfelis/Facio.html and http://dasher.wustl.edu/tinker/) [27, 28]. The dimension of the peptide monomer was calculated using Swiss-PdbViewer (http://www.expasy.org/spdbv/) [29] (Fig. 1A).

2.3. Gel stretch chamber

In this study, a gel stretch chamber was employed for the three-dimensional cell culture and the tension experiments. The gel stretch chamber was constructed by attaching a piece of silicone foam sheet (SPP-2.0S, AS ONE, Osaka, Japan) that had been manually cut to a size of approximately 20 mm × 4.5 mm × 2 mm (length × width × thickness), with a rectangular hole of approximately 18 mm × 1.5 mm (length × width) for holding the peptide gel, to the inner wall of a commercially available stretch chamber (STB-CH-04, STREX, Osaka, Japan). Silicone resin (TSE3032, GE Toshiba Silicones, Tokyo, Japan) was used as a glue. The silicone foam sheet was located approximately 2 mm above the bottom of the chamber to allow the cell culture medium to contact the bottom of the hydrogel. A hydrophilic surface treatment, which consisted of high power plasma sterilization (PDC-32G, Harrick Scientific Products Inc., NY, USA) for 10 minutes, was performed to increase the hydrophilicity of the silicone foam sheet surface before its use.

2.4. Attenuated total reflection-Fourier transform infrared spectroscopy (ATR- FTIR)

The sample for the ATR-FTIR was prepared by dissolving SPG-178 peptide powder in a deuterated aqueous solution at a final concentration of 1 % (w/v). The pH of the sample was adjusted to approximately pH=6.5 by adding aliquots of a 0.5 % (w/v) sodium hydrogen carbonate solution. The sample formed a hydrogel at this condition (1 % [w/v] at pH=6.5), which indicated the presence of nanofibers and the

three-dimensional network structure formation of the SPG-178 peptide. The spectrum was recorded on a Perkin-Elmer Spectrum One spectrometer equipped (Perkin-Elmer, Norwalk, CT, USA) with a Horizontal ATR (HATR) Sampling Accessory and a trough plate, which was comprised of a ZnSe crystal with a 45 ° angle of incidence. One milliliter of the hydrogel was spread directly onto the surface of the trough plate. The spectrum was recorded at room temperature from 4000 cm^{-1} to 650 cm^{-1}, and 32 scans were collected with a spectral resolution of 4 cm^{-1}. A deuterated water spectrum was used as background and was subtracted from the sample spectrum.

2.5. Transmission electron microscopy (TEM)

Dulbecco's modified Eagles medium (DMEM; WAKO, Osaka, Japan) was mixed with the peptide solution described in section *2.1* at a volume ratio of 1:2. The mixture formed a hydrogel due to the increased salt concentration [14, 15, 30] even though the peptide concentration was lowered from 2.4 mM to 1.6 mM. The final concentration of the SPG-178 peptide in the hydrogel was 1.6 mM. A 60 μl aliquot of the hydrogel was added to the rectangular hole in the silicone foam sheet in the gel stretch chamber. The chamber was filled with 3 ml of DMEM that was supplemented with 10 % (v/v) fetal bovine serum (FBS) and incubated in 5 % CO_2 at 37 °C for 8 days. After the incubation, the DMEM supplemented with 10 % (v/v) FBS was removed, and the hydrogel was fixed in a 0.1 M phosphate buffer containing 2 % (v/v) glutaraldehyde and 2 % (v/v) paraformaldehyde at 4 °C over night and postfixed in 2 % (w/v) osmium tetroxide for 2 hours at room temperature. The SPG-178 hydrogel was dehydrated using graded concentrations of ethanol. After dehydration, the hydrogel was embedded in Spurr resin (Polysciences, Warrington, PA, USA). Ultrathin sections (60-90 nm) were cut with a Leica EM UC6 ultramicrotome (Leica, Vienna, Austria) and stained with uranyl acetate

and lead citrate. Visualization was performed using a Hitachi 7650 electron microscope (Hitachi, Tokyo, Japan), which was operated at 80 kV.

2.6. Rheology measurement

The SPG-178 hydrogel, which contained 1.6 mM of the SPG-178 peptide was prepared as described in section *2.5.* A 40 µl aliquot of the hydrogel was placed on the plate of a rheometer (AR1000, TA Instruments, New Castle, DE, USA). A 20-mm-diameter, 1 ° aluminum cone with truncation at 24 µm was lowered so that the tip was 24 µm above the plate. A solvent trap was used to maintain a water-saturated atmosphere to prevent the evaporation of solvent during the measurement. The SPG-178 hydrogel was tested over a range of frequencies from 10 to 0.1 rad/s at 1.0 µNm oscillatory torque to measure the storage modulus (*G′,* the elastic response) and the loss modulus (*G″*, the viscous response) at 37 °C. As controls, a 2.4 mM SPG-178 peptide solution and DMEM were tested in the same manner.

2.7. Three-dimensional cell culture

Murine C2C12 myoblasts (ECACC: 91031101) were obtained from the European Collection of Cell Cultures (DS Pharma Biomedical, Osaka, Japan). The C2C12 cells were cultured on a Petri dish and grown until 50 % confluence in DMEM supplemented with 10 % (v/v) FBS in 5 % CO_2 at 37 °C. To start the three-dimensional cell culture, the cells were trypsinized and suspended in DMEM and then mixed with a 2.4 mM SPG-178 peptide solution at a volume ratio of 1:2. The final concentration of the cells and the peptide in the hydrogel were 2×10^6 cells/ml and 1.6 mM, respectively. A 60 µl aliquot of the hydrogel was added in the rectangular hole of the silicone foam sheet in the gel stretch chamber. For the cell proliferation assay, the same amount of the

hydrogel was added to a 1.5 ml tube and stored at -80 °C (day 0 control). The chamber was filled with 3 ml of DMEM that was supplemented with 10 % FBS and incubated in 5 % CO_2 at 37 °C. The medium was replaced with 3 ml of fresh medium once every 2-3 days.

2.8. Live and dead assay

C2C12 cells were cultured in the SPG-178 hydrogel as described in section *2.7*. On the 8th day of incubation, the medium was changed to DMEM. Calcein (calcein-AM, Dojindo, Kumamoto, Japan) and 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Dojindo) were add to the gel stretch chamber at a final concentrations of 10 μM to stain the nuclei of live and dead or injured cells. After 30 minutes of incubation in 5 % CO_2 at 37 °C, the stained cells that were located approximately 500 μm above the bottom of the hydrogel were observed on a confocal laser scanning microscopy system (ex/em=405/460 nm for DAPI and 488/515 nm for calcein, FV-1000, Olympus, Tokyo, Japan).

2.9. Cell proliferation assay

C2C12 cell proliferation in the SPG-178 hydrogel was measured with a CyQUANT Cell Proliferation Assay Kit (C7026, Molecular Probes, Eugene, OR, USA) with small modifications to the manufacturer's protocol. The hydrogels described in section *2.7* were transferred from the gel stretch chamber to a 1.5 ml tube after 2, 4, 6, and 8 days of incubation and were stored at -80 °C for at least one hour before the assay. After collecting and storing all of the samples, the hydrogels, including the day 0 control described in section *2.7,* were thawed and dispersed into 940 μl of Cell-Lysis Buffer (1X component B). Ten microliters of each dispersed gel solution were transferred to a

well of a 96-well plate. Two hundred microliters of CyQUANT GR dye solution (a mixture of 1X component A and 1X component B) were added to each well and measured on a spectrofluorometer with the FLUO star OPTIMA software. The number of cells in the gel mixture was extrapolated from a standard curve that was generated with known amount of C2C12 cells over a range of 6×10^2 cells – 2.4×10^4 cells per well.

2.10. Stretching cells in the SPG-178 hydrogel

C2C12 cells were cultured in the peptide gel as described in section *2.7*, with the exception of changing the SPG-178 hydrogel volume from 60 µl to 90µl. The volume increase allowed for the deep intrusion of the hydrogel into the cavities of the silicone foam, which resulted in the prevention of the detachment of the hydrogel during the stretch. The gel stretch chamber was placed into a hand-control stretch device (STB-10, STREX). On the 5th day of incubation, the cells in the hydrogel were stained by adding calcein-AM in the chamber at a final concentration of 10 µM. After 30 minutes of incubation, the chamber was statically stretched by 20 %. The stained cells that were located approximately 500 µm above the bottom of the hydrogel were observed on the confocal laser scanning microscopy system FV-1000 before and after the stretch.

2.11. Static stretch for ERK activation

C2C12 cells were cultured in the peptide gel as described in section *2.10.* On the 8th day of incubation, the medium was changed from DMEM that was supplemented with 10 % FBS to a low serum differentiation medium (DMEM supplemented with 2 % [v/v] horse serum). After an additional 2 days of incubation, the gel stretch chamber was statically stretched by 10 % for 5 minutes in 5 % CO_2 at 37 °C (Stretched sample;

ST). Parallel sets of non-stretched C2C12 cells that were cultured in the SPG-178 hydrogels in the gel stretch chamber were used as a control (Non-stretch control; NST). MEK1 inhibitor (PD98059, Cell Signaling Technology, Beverly, MA, USA) that was dissolved in DMSO was added to the gel stretch chamber as necessary to achieve a final concentration of 50 μM one hour before the stretch (Stretched sample with PD98059; STP). The control samples for the inhibitor were treated with the same volume of vehicle DMSO (Stretched sample with DMSO; STD). After the completion of the stretch, the SPG-178 hydrogel was collected for Western Blotting.

2.12. Western Blotting

The SPG-178 hydrogels were transferred to a 1.5 ml tube that contained 1 ml of ice-cold TBS with protease and phosphatase inhibitors (1 % [v/v], 78440, Thermo Fisher Scientific, Rockford, IL, USA) to remove any excess medium in and on the gel. The tube was centrifuged at 1,200 ×g (3,550 rpm) for 5 minutes at 4 °C, the supernatant was discarded, and 100 μl of RIPA lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA) with the protease and phosphatase inhibitors were added. The cell lysate was sonicated for a total of 25 sec with a sonicator (BRANSON Digital Sonifier II, BRANSON, Danbury, CT, USA) to break down the gel fiber structure and was cleared at 21,500 ×g (15,000 rpm) for 45 minutes at 4 °C. The supernatants were transferred into new tubes and quantified using the BCA protein assay kit (Piece, Rockford, IL, USA). Approximately 25 μg of protein were loaded in a 10 % SDS polyacrylamide gel (SDS–PAGE) and transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). The membrane was blocked in 10 % Blocking One (Nacalai Tesque, Kyoto, Japan) in TBS and incubated with primary antibodies: anti-ERK (4695, 1:1,000, Cell Signaling Technology), and anti-Phosphorylated ERK (Thr202/Tyr204;

4370, 1:1,000, Cell Signaling Technology). The blots were developed by chemiluminescence using LumiGLO (Cell Signaling Technology) to quantify the relative intensities (RI) and a tetramethylbenzine (TMB) solution (EzWestBlue, Atto, Tokyo, Japan) to show a representative blot.

3. Results

3.1 ATR-FTIR

The existence of a β-sheet structure within the hydrogel was supported by ATR-FTIR spectroscopy (Fig. 2A). The spectra showed predominant peaks at 1,616 cm^{-1}, which indicated the presence of aggregated β-sheets. The typical amide groups containing a β-sheet structure give rise to peaks ranging from 1,620 cm^{-1} to 1,640 cm^{-1}. The small peak at 1,679 cm^{-1} suggested the presence of antiparallel β-sheets in the nanofiber structure of the SPG-178 hydrogel. In contrast, there was no peak regarding helical character (1,640-1,660 cm^{-1}). Trace amounts of TFA accounted for the peak observed at 1,672 cm^{-1} in the spectrum.

3.2. TEM

The TEM image from the ultrathin layer of the SPG-178 hydrogel showed the nanofibers and the network structure in the hydrogel (Fig. 2B). The diameter of the nanofibers was estimated to be less than 10 nm, which corresponds to the calculated length of the SPG-178 peptide monomer in β-sheet form (Fig. 1A). Partial mesh structures that were formed by the nanofibers were observed. The mesh size was variable, reaching up to 500 nm.

3.3. Mechanical properties of the SPG-178 hydrogel

Frequency sweep measurements of the SPG-178 hydrogel (1.6 mM) showed that the storage modulus (G', the elastic response) and the loss modulus (G'', the viscous response) values were relatively constant and that the G' values were much greater than zero. In addition, the G' values over the entire frequency range exceeded those of G''. This result reflected the gel-like property of the SPG-178 hydrogel [26, 30]. In contrast, the G' and G'' values obtained from the measurement with the DMEM sample, used as a representative example of a liquid, were low and decreased in an oscillatory manner. The result from the measurement with a 2.4 mM SPG-178 peptide solution was relatively similar to that of the DMEM sample and demonstrated a liquid-like property [26, 30]. The G' and G'' values were very close at each oscillatory frequency.

3.4. Live and dead, and cell proliferation assays.

In the live and dead assay, the extended shapes of the C2C12 cells that were stained with calcein and a certain number of the nuclei stained with DAPI were observed in the SPG-178 hydrogel (Fig. 4A-C). Whereas the live/dead cell ratio of the observed area was lower than that of the surface area of the hydrogel (data not shown), the extended cells clearly showed that the hydrogel provided a suitable interface for the cells to adhere and survive. C2C12 cell proliferation in the SPG-178 peptide hydrogel scaffold was monitored by conducting DNA analysis for up to 8 days. As shown in Fig. 4D, the C2C12 cells proliferated gradually through 8 days of culture. Based on the measured DNA content, the estimated number of C2C12 cells in the hydrogel had increased by 12.4 ± 1.5 (mean ± SEM, n=4) times by the end of the incubation period.

3.5. Stretching cells in the SPG-178 hydrogel

The elongation of C2C12 cells in the SPG-178 hydrogel was observed as the gel stretch chamber was stretched by 20 % with the hand-control stretch device STB-10 (Fig. 5). The distance between the asterisks was 260 μm before the stretch (Fig. 5A) and 310 μm after the stretch (Fig. 5B). The calculated ratio of the increased in distance was 19 %, which corresponded to the stretch ratio of the gel stretch chamber. The successful cell stretch in the hydrogel indicated the deep intrusion of the SPG-178 hydrogel into the cavities of the silicone foam; therefore, the hydrogel was able to transmit the mechanical stress to the cells that were incorporated into the hydrogel. This result proved the usability of the stretch system for three-dimensionally cultured cells that consisted of the SPG-178 hydrogel, the gel stretch chamber, and the hand-control stretch device STB-10.

3.6. ERK phosphorylation by mechanical stimulation.

Western blot analysis revealed that ERK in the three-dimensionally cultured C2C12 was activated by a static stretch (Fig. 6). The degree of the ERK phosphorylation was increased by 2.8 ± 0.2 (mean ± SEM, n=15) times by the 5-minute stretch (ST) compared to the untreated control basal value (NST: 1.0 ± 0.1, mean ± SEM, n=8). The addition of 50 μM PD98059 completely inhibited the stretch-induced phosphorylation of ERK (STP), whereas the addition of DMSO, a solvent of PD98059, did not significantly affect the ERK phosphorylation (STD). The degree of the ERK phosphorylation was 0.6 ± 0.1 (mean ± SEM, n=8) in STP and 2.3 ± 0.3 (mean ± SEM, n=7) in STD. The blocking effect of the PD98059 emphasized the effect of the brief stretch on the ERK phosphorylation in three-dimensionally cultured cells. No change in the total ERK protein concentration was observed in any sample.

4. Discussion

In previous studies, self-assembling peptides with a total net charge of +2 have been reported to successfully form a hydrogel at pH=7.4 [26, 31]. The SPG-178 peptide solution (2.4 mM) is transparent and able to form a stable hydrogel at neutral pH when it is triggered by an increase in salt concentration (Fig. 1B). The stability of the peptide solution/hydrogel at neutral pH contributes to the biocompatibility of the scaffold and provides an additional benefit for the sterilization procedure. In fact, the SPG-178 solution at neutral pH can be sterilized with an autoclave. Insignificant degradation of the SPG-178 peptide was detected with MALDI-TOFF MASS (see Fig. S1 and S2), and no change in the gelation behavior was caused by autoclaving. In contrast, collagen scaffolds require relatively complicated procedures and expensive equipment, such as ethylene oxide gas treatment and γ-irradiation due to thermal denaturation [32]. The decomposition of the conventional self-assembling peptide RADA-16 provided with low pH (Puramatrix, Becton Dickinson, CA, USA) by autoclaving was confirmed by MALDI-TOFF MASS (see Fig. S3 and S4)

In the structural studies, the ATR-FTIR spectra shown in Fig. 2A indicated the presence of considerable anti-parallel β-sheet content with no or negligible helical component in the SPG-178 hydrogel, as has been observed for conventional self-assembling peptides [16]. The TEM image from the ultrathin layer of the SPG178 hydrogel demonstrated the nanofiber structure of the self-assembled peptide with a diameter of less than 10 nm. Taken together, the gelation process of the SPG-178 peptide was confirmed to correspond with the molecular models shown in Fig. 1. The partial mesh structure that was formed by the peptide nanofibers was observed in the

TEM image. Some of the other nanofibers that seemed too short to form the mesh structure were considered to have been cut in the procedure for preparing the ultrathin layer. The average length of the nanofiber was difficult to be estimated from the image for the same reason. However, the mesh size (approximately 500 nm) of the SPG-178 hydrogel was considered to be much smaller than the size of the cells (approximately 10 μm). Since, the SPG-178 peptide nanofibers are not chemically cross-linked, the mesh structure of the hydrogel can easily change. Thus, the pore size of the hydrogel is considered to widen and be rebuilt as cells in the hydrogel infiltrate or ingrow.

The gel-like property of the SPG-178 hydrogel was demonstrated with the rheology measurement. Both the storage modulus, G', and the loss modulus, G'', of the hydrogel were relatively low compared to other self-assembling peptides [33]. This can be explained by the lower concentration of the SPG-178 in the hydrogel (0.27 % [w/v]) compared to the other approximately 2 % (w/v) hydrogels. The low mechanical strength of the SPG-178 hydrogel contributes to the homogeneous distribution of the cells in the mixing process at the beginning of the cell culture. Next, the further increase in the salt concentration during the immersion of the SPG-178 hydrogel into the cell culture medium will enhance the gelation to increase the mechanical strength of the hydrogel.

In the live and dead assay, the extended shapes of the C2C12 cells were observed in the SPG-178 hydrogel, which indicated tight cell adhesion onto the peptide nanofiber. In addition, the DNA content measurement revealed that the three-dimensionally cultured C2C12 cells proliferated successfully during the incubation. These results indicate that the SPG-178 hydrogel is suitable as a scaffold. According to the mesh size (approximately 500 nm) of the SPG-178 observed by TEM, the cells must be suspended in the three-dimensional nanofiber network of the SPG-178 hydrogel. The charged

amino acid residues within the peptide nanofiber, especially the positively charged arginine residues, are considered to support cell adhesion at the beginning of the culture [34, 35]. The serum proteins in the cell culture medium may also attach to the peptide nanofiber and help the cell adhesion [36]. Furthermore, there are studies introducing a cell adhesion motif such as RGD in self-assembling peptide hydrogels to improve cell adhesion and survival ratios [37, 38]. These techniques might also be useful for the SPG-178 hydrogel. In other studies using rat skeletal muscle cell (L6) and human chondrosarcoma cells (OUMS-27), accumulated type-I collagen and aggrecan in the SPG-178 hydrogel were observed, respectively (unpublished data). These secreted and accumulated ECM components in the hydrogel are expected to contribute to the cell proliferation and migration. Further research is needed to understand the detailed mechanism of cell adhesion, extension, and migration in SPG-178 hydrogels.

The confocal microscope observations showed the elongation of the cells and the increased distance between the cells as the peptide gel was stretched. These results proved that the peptide gel scaffold was “stretchable” and capable of transmitting the mechanical stimulation to the inner cells. The plasma-treated hydrophilic surface of the silicone foam sheet and the low mechanical strength (low viscosity) of the SPG-178 hydrogel facilitated the deep infiltration of the hydrogel into the cavities of the silicone foam. Thus, the expanded area of contact between the silicone foam sheet and the hydrogel produced a friction force that was great enough to stretch the hydrogel without slipping. Consequently, the stretch system that consisted of the SPG-178 hydrogel, the gel stretch chamber, and the hand-control stretching device STB-10 was confirmed to be usable for the stretching of three-dimensionally cultured cells.

Stretch-induced ERK phosphorylation have been previously shown in many papers including studies in the two-dimensional cell culture of C2C12 cells [39, 40], fibroblast

cells [41-43], human keratinocytes [44], and whole rat skeletal muscle stretching [45]. In these studies, the peak time of the ERK phosphorylation varied because of the different cell cultures and stretch systems. However, generally, the ERK phosphorylation started in the early stage of the stretch induction in these studies. In our study, the ERK in the C2C12 cells were also activated quickly by stretching in the SPG-178 hydrogel. The transmission of the mechanical stimulation was supported by the friction force between the gel stretch chamber and the SPG-178 hydrogel and the cell adhesion to the peptide nanofiber network. The gel stretch chamber can be incorporated in an automatic stretch system suitable for cyclical stretching or a long-term stretching experiment to investigate the cellular response to mechanical stress in three-dimensional cell culture.

5. Conclusions

Mechanical stimulation is now widely incorporated into three-dimensional cell culture systems for tissue engineering. We studied the biocompatibility and the ability to transmit mechanical stimulation of a self-assembling peptide hydrogel. The self-assembling peptide SPG-178 was 100 % chemically synthesized and was confirmed to form an anti-parallel β-sheet structure in aqueous solution. The peptide self-assembled to form a nanofiber with a diameter of less than 10 nm, which further formed a hydrogel. The results of the three-dimensional cell culture clarified that the developed self-assembling peptide gel was non-cytotoxic. Western Blot analysis demonstrated the rapid phosphorylation of ERK induced by the static stretching of the hydrogel. These results indicated that the self-assembling peptide hydrogel was a suitable tool for the investigation of the effect of mechanical stress on

three-dimensional cell culture. Additional studies are needed for a better understanding of the contribution from the secreted ECM component.

6. Acknowledgements

We thank Ms. Masumi Furutani for her efficient technical support in obtaining the TEM images and Dr. Ken Takahashi for the helpful discussions on the experimental details. We are indebted to Dr. Yasuyuki Ishida at Chubu University for generously allowing us use of a MALDI-TOF mass spectrometer. This work was partially supported by Grant-in-Aid for Scientific Research of Priority Areas, 17076006 provided by the Ministry of Education, Culture, Sports, Sciences, and Technology of Japan (to K.N.), and Challenging Exploratory Research, 23650264 provided by the Japanese Society for the Promotion of Science (to K.N.). Y.N. and H.Y. were supported by Menicon Co., Ltd., Japan.

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Figure Captions

Figure 1

The self-assembling peptide SPG-178 hydrogel scaffold. (A) A molecular model of SPG-178, the dimensions of which are 47.5 ×12.8 ×3.1 Å. For the representation, Facio was used: cyan, carbon; red, oxygen; blue, nitrogen; white, hydrogen. (B) A schematic diagram of the formation of the hydrogel from the peptide monomer.

Figure 2

The structural properties of SPG-178. (A) ATR-FTIR spectra of the SPG-178 peptide solution. (B) A TEM image of the peptide nanofibers in the hydrogel. The scale bar is 200 nm.

Figure 3

The mechanical properties of the SPG-178 hydrogel. (●) SPG-178 hydrogel 0.27 % (w/v) *G'*, (○) SPG-178 hydrogel 0.27 % (w/v) *G''*, (▲) SPG-178 solution 0.4 % (w/v) *G'*, (Δ) SPG-178 solution 0.4 % (w/v) *G''*, (■) DMEM *G'*, (□) DMEM *G''*. Bars represent the mean ± SEM. (n=3)

Figure 4

Live and dead assay and cell proliferation assay. C2C12 cells in a SPG-178 hydrogel were stained with A) DAPI and B) calcein. The merged image is shown in (C). The proliferation of the C2C12 cells in the peptide gel was estimated by measuring DNA content (D). Scale bar shown in (A) is 50 μm. Bars represent mean ± SEM. (n=4)

Figure 5

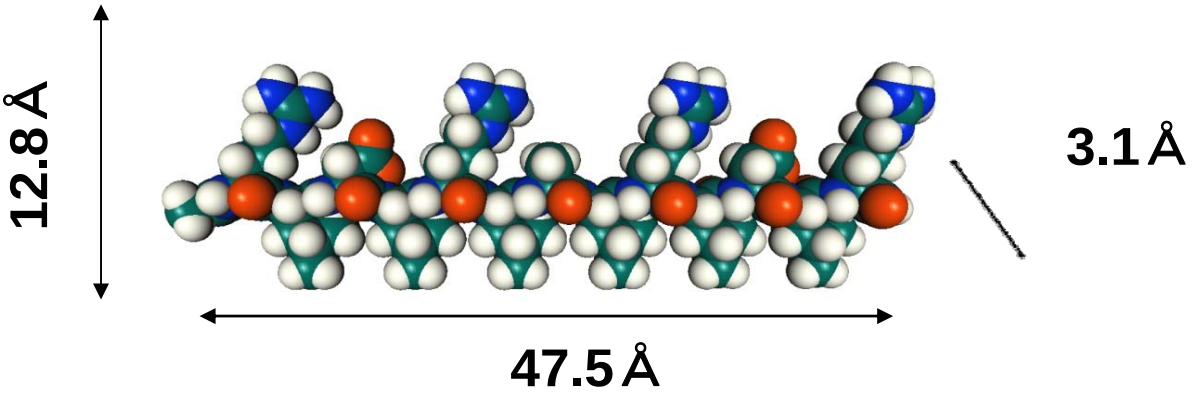
C2C12 cells in the peptide gel stained with calcein-AM (A) before and (B) after the stretch (20 %) in the stretch chamber. The intercellular distance between the asterisks was 260 μm before the stretch and 310 μm after the stretch.

Figure 6

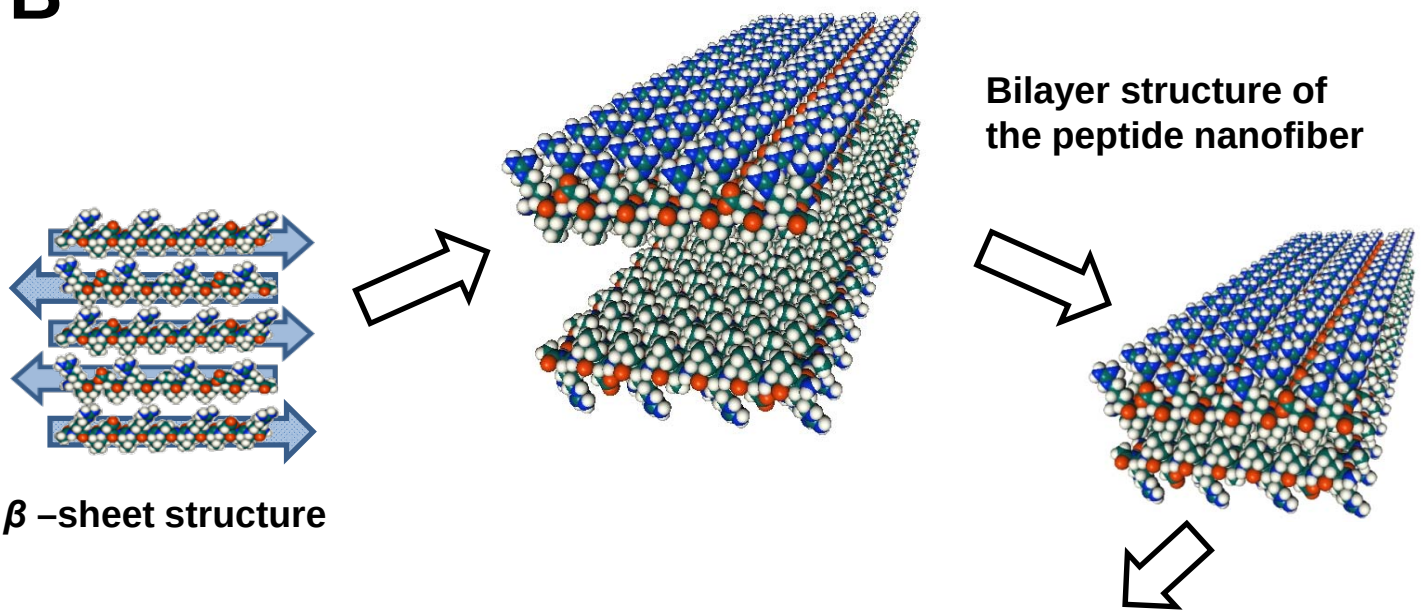
Western Blot analysis. A representative blot of phosphorylated ERK (p-ERK1/2) and total ERK (ERK1/2) showed an increased intensity in labeling of phosphospecific ERK antibody following the stretch, and the effect of the MEK inhibitor PD98059. Bars represent mean ± SEM (n=8 (NST), 15 (ST), 7 (STD), 8(STP))

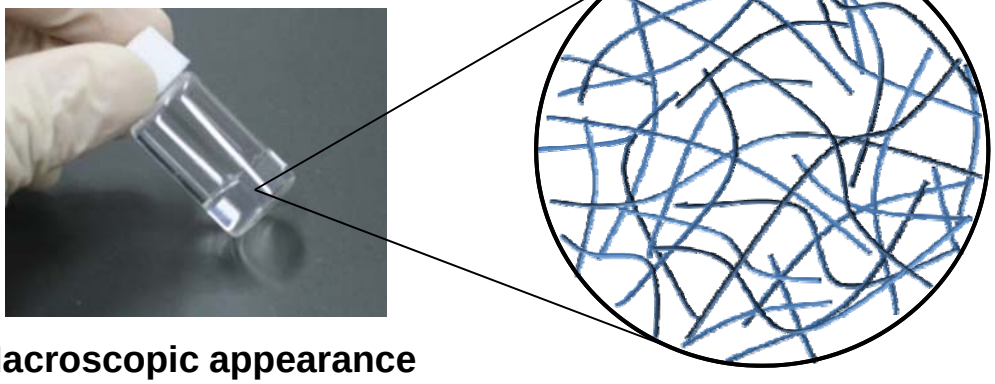
A

RLDLRLALRLDLR



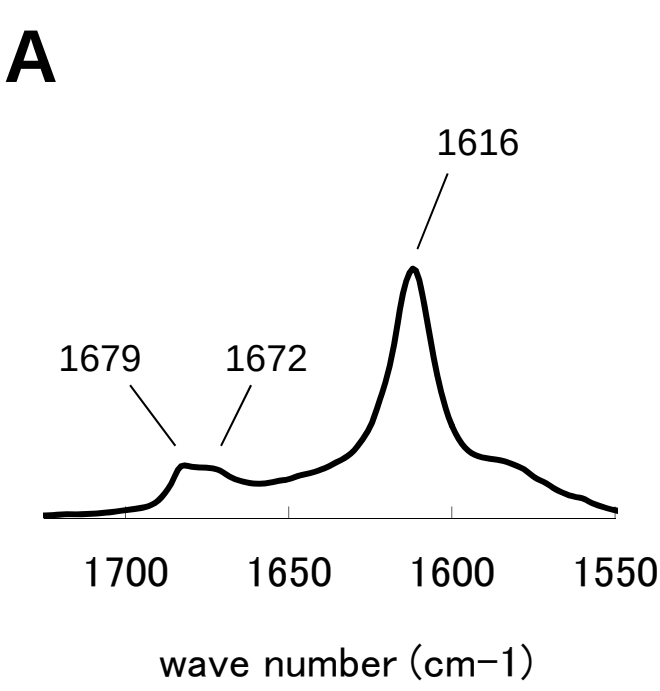
B

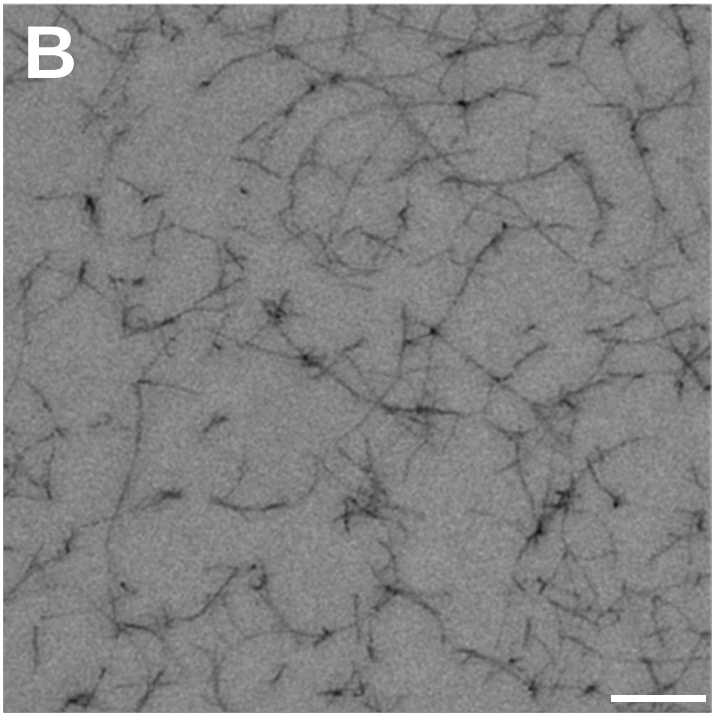




Macroscopic appearance
of the peptide hydrogel

3D mesh structure


A
1616
1679
1672
1700
1650
1600
1550
wave number (cm−1)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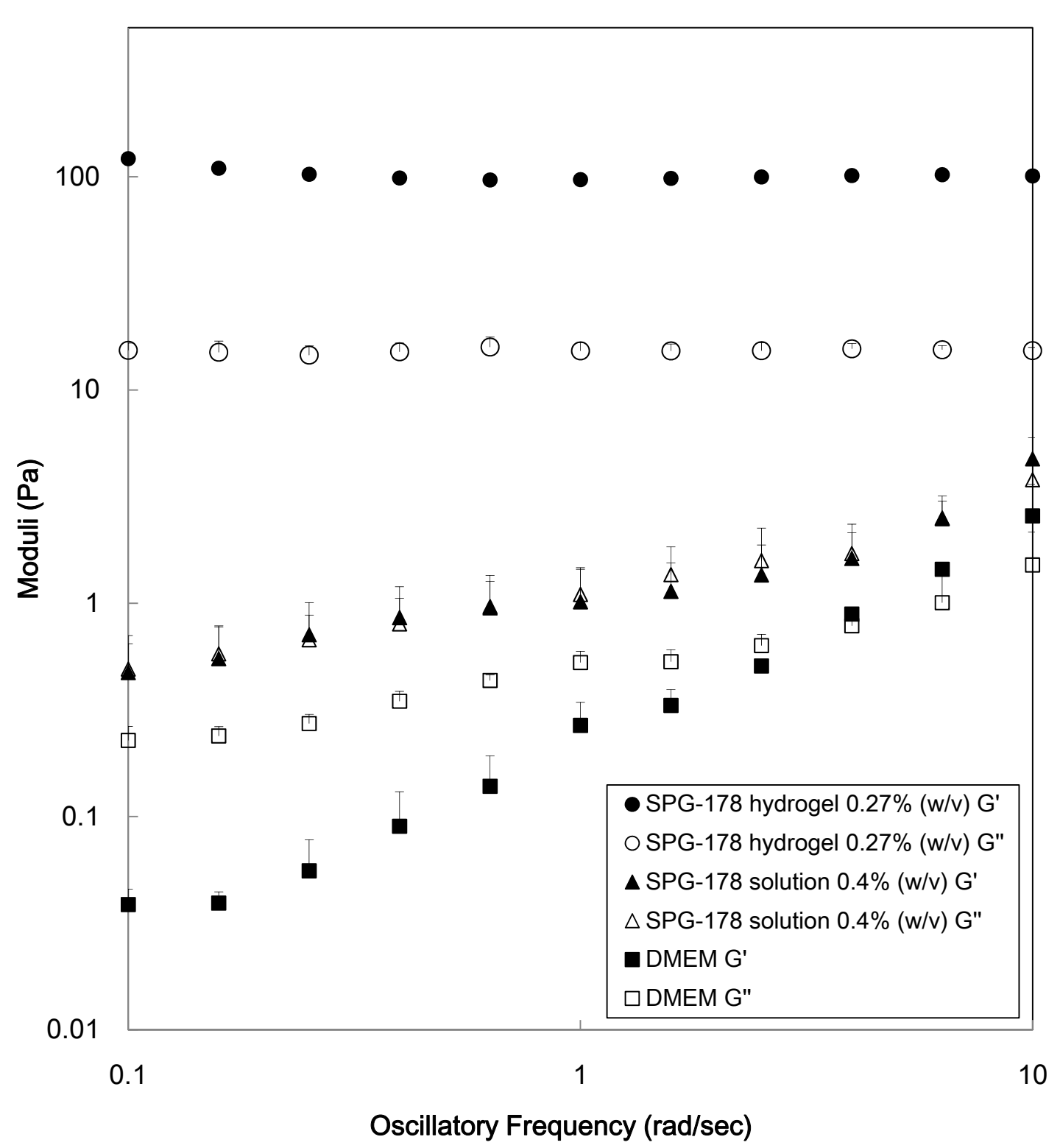
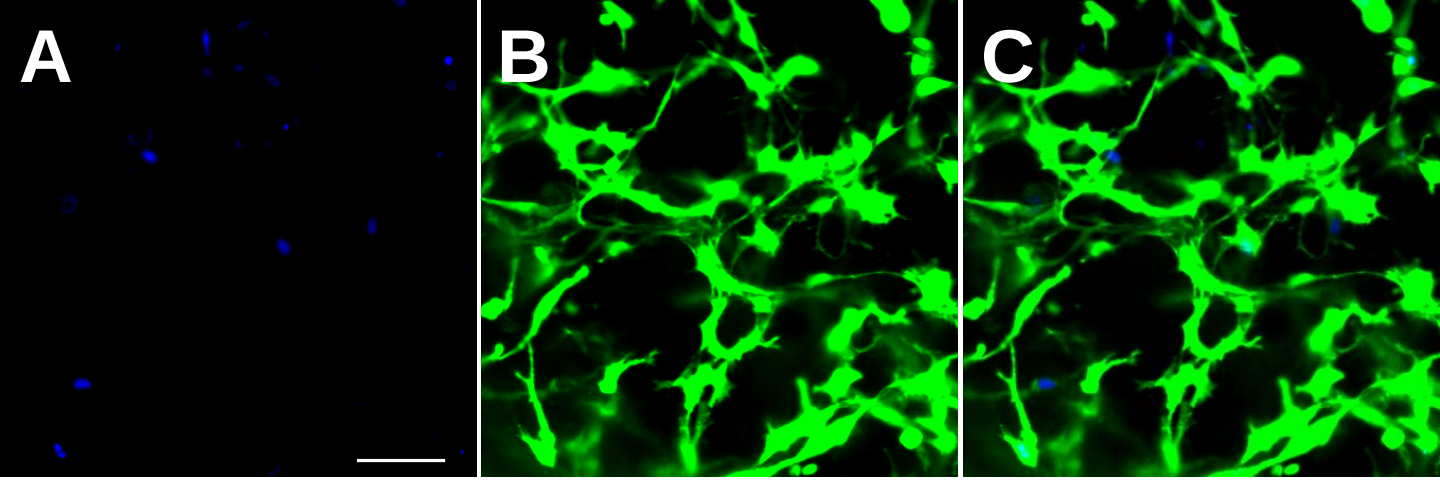
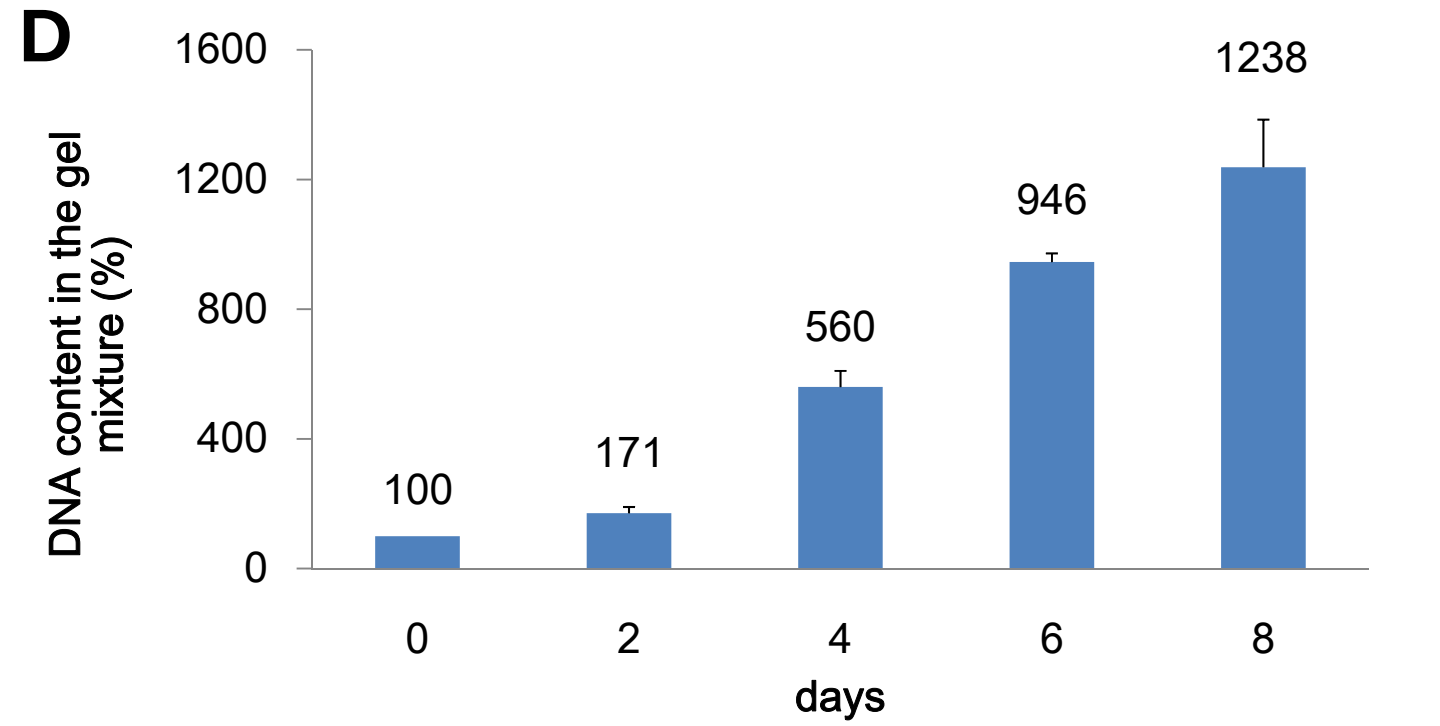
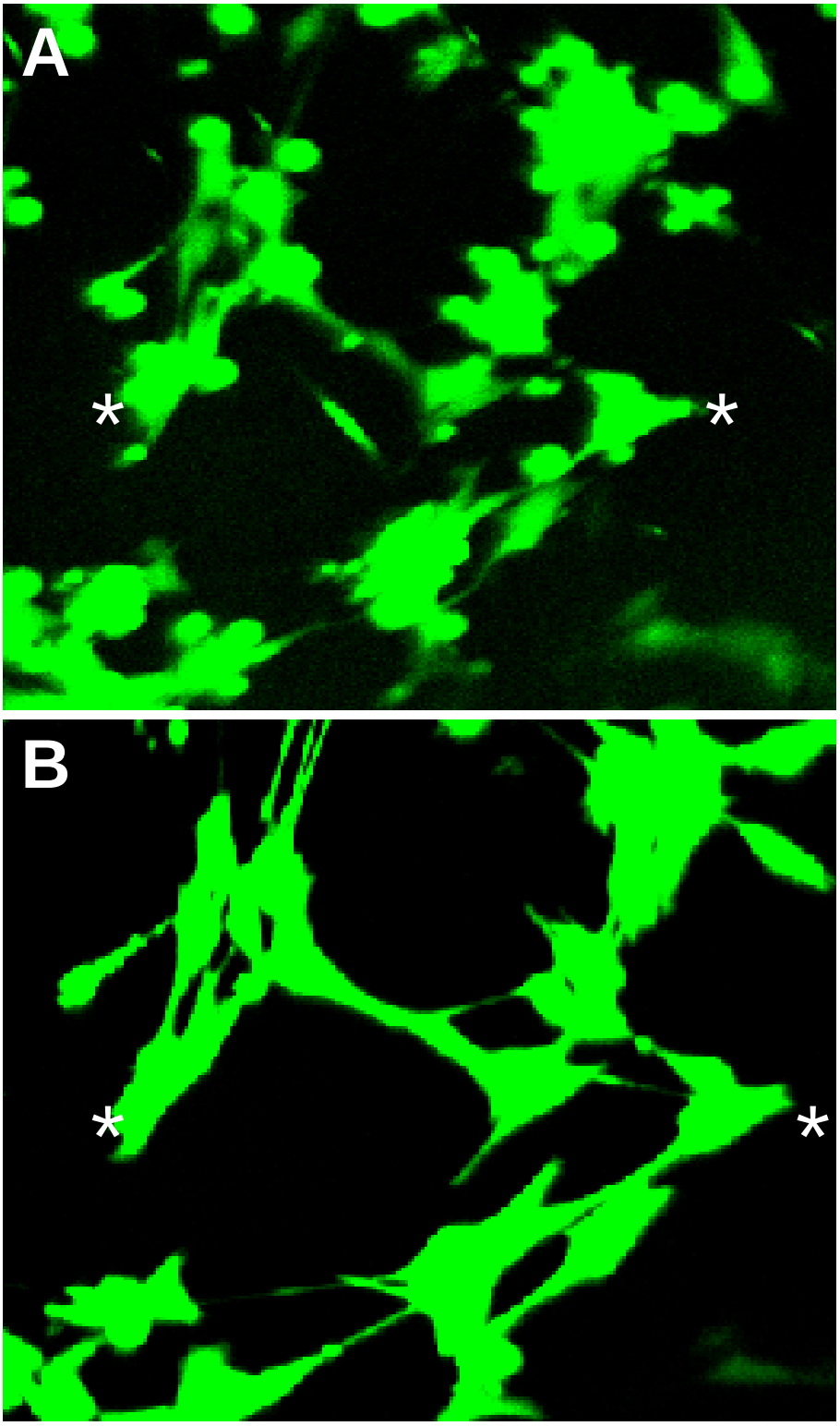

100
10
1
0.1
0.01
Moduli (Pa)
0.1
1
10
Oscillatory Frequency (rad/sec)
SPG-178 hydrogel 0.27% (w/v) G'
SPG-178 hydrogel 0.27% (w/v) G"
SPG-178 solution 0.4% (w/v) G'
SPG-178 solution 0.4% (w/v) G"
DMEM G'
DMEM G"

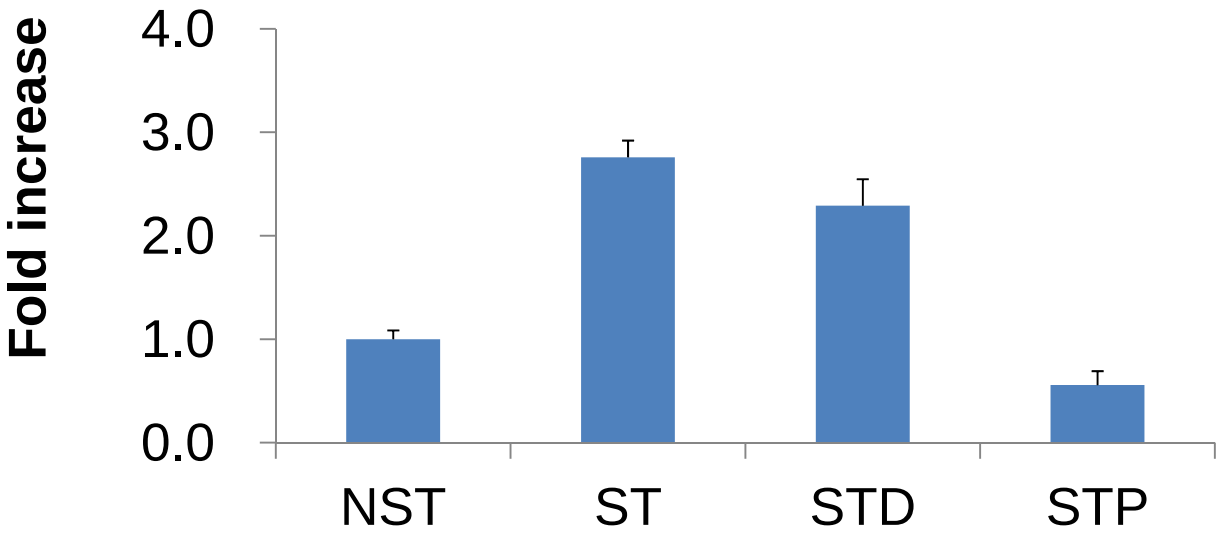

A
B
C


D
DNA content in the gel mixture (%)
1600
1200
800
400
0
100
171
560
946
1238
0
2
4
6
8
days


A
*
*
B
*
*

p-ERK 1/2

ERK 1/2



Stretch	-	+	+	+
DMSO	-	-	+	+
PD98059	-	-	-	+

Supplementary Data

Peptide Synthesis

Peptide SPG178 was manually synthesized by a standard solid phase method using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on a KMS-3 peptide synthesizer (Kokusan chemical Co. Ltd., Tokyo, Japan). Fmoc-protected amino acids (Fmoc-Arg[Pbf], Fmoc-Ala·H_2O, Fmoc-Leu, Fmoc-Asp[OBu^t]) were purchased from the Peptide Institute, Inc. (Osaka, Japan). The peptide was prepared on CLEAR^{TM}-amide resin (Peptide Institute) in dimethylformamide (DMF, Sigma-Aldrich Japan, Tokyo, Japan) via 1-hydroxybenzotriazole hydrate/N,N'-diisopropylcarbodiimide (Watanabe Chemical Industries, Ltd., Hiroshima, Japan) activation. The Fmoc protecting group was deprotected by 20 % piperidine (Watanabe Chemical Industries, Ltd.) in DMF. To protect the N-terminal of the peptides by an acetyl group, 10 equimolar amounts of acetic anhydride (Nacalai Tesque) were reacted for 2 hours in DMF. To cleave the peptide from the solid support, it was treated in a mixture of trifluoroacetic acid (TFA, WAKO), 1,2-ethanedithiol (Tokyo Chemical Industry, Ltd., Japan), thioanisole (Tokyo Chemical Industry, Ltd.), triisopropylsilane (Watanabe Chemical Industries, Ltd.), and Milli-Q water (Millipore) in a ratio of 82:6:6:3:3 for 3 hours at room temperature. The cleavage mixture was added into an excess amount of cold diethylether (WAKO), and the peptide was precipitated. The white precipitation was collected by centrifugation, washed with cold ether, and air dried. The peptide powder was dissolved in 5 mM hydrochloric acid (Nacalai Tesque) and freeze-dried. Further purification was not performed.

Mass Spectroscopy

The molecular weight of the peptide SPG178 and the Puramatrix was measured by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) using an Autoflex III (Bruker Daltonics, Bremen, Germany). The peptide was dissolved in a 0.1 % TFA aqueous solution at a concentration of 0.1 %, and α-cyano-4-hydroxycinnamic acid (WAKO) was dissolved in 0.1 % TFA/acetonitrile (WAKO) (1:1) at a concentration of 1 %. A mixture of 1 μL of peptide solution and 1 μL of matrix solution was placed on a MTP 384 target plate ground steel T F (Bruker Daltonics) and air dried. The mass spectra were recorded in positive ion mode and the average mass was determined as $[M+H]^+$.

Supplementary Data Figure Captions

Figure S1

MALDI-TOF-MS spectrometry of the SPG178 peptide for the molecular weight measurement

Figure S2

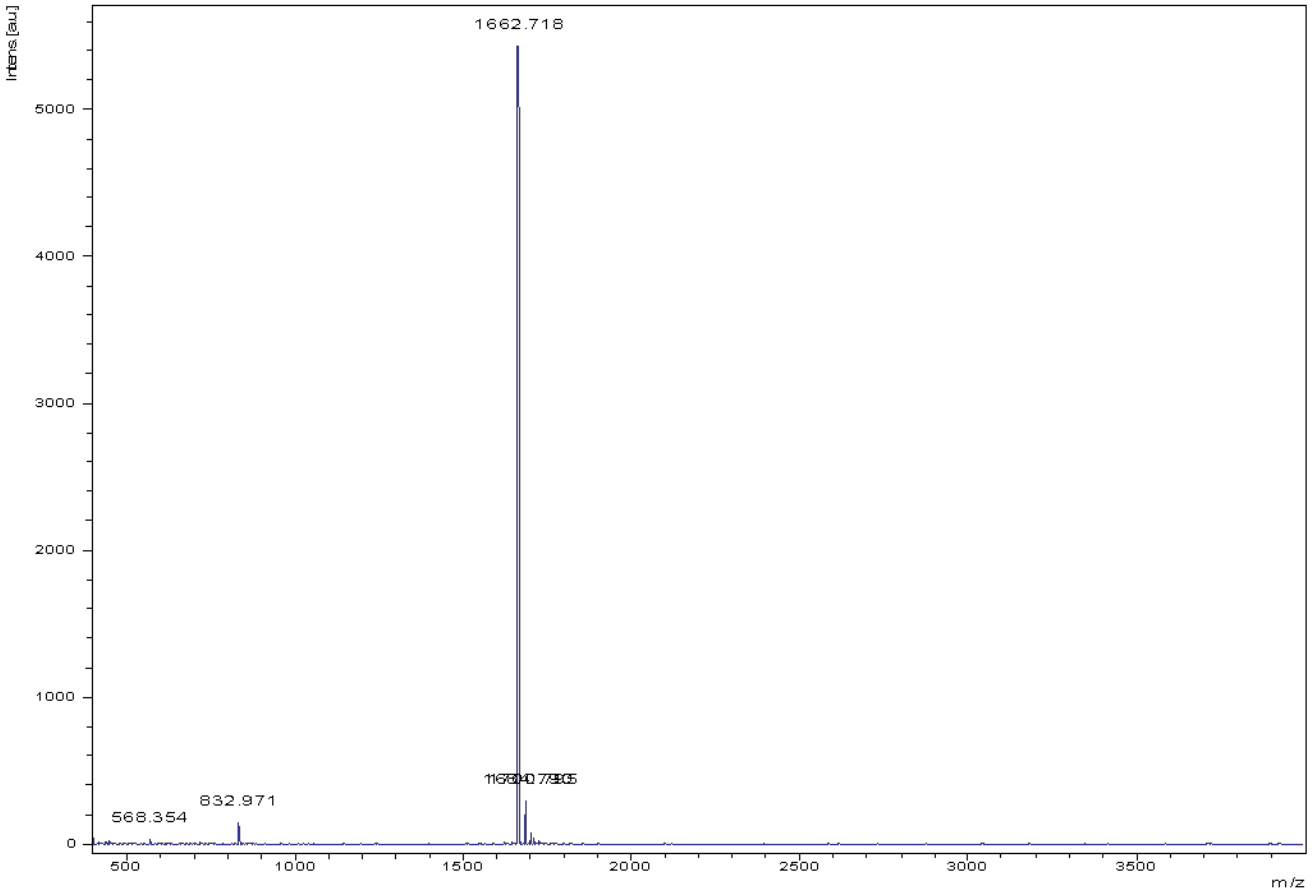
MALDI-TOF-MS spectrometry of the SPG178 peptide after autoclaving

Figure S3

MALDI-TOF-MS spectrometry of Puramatrix before autoclaving

Figure S4

MALDI-TOF-MS spectrometry of Puramatrix after autoclaving


Intens.[a.u.]
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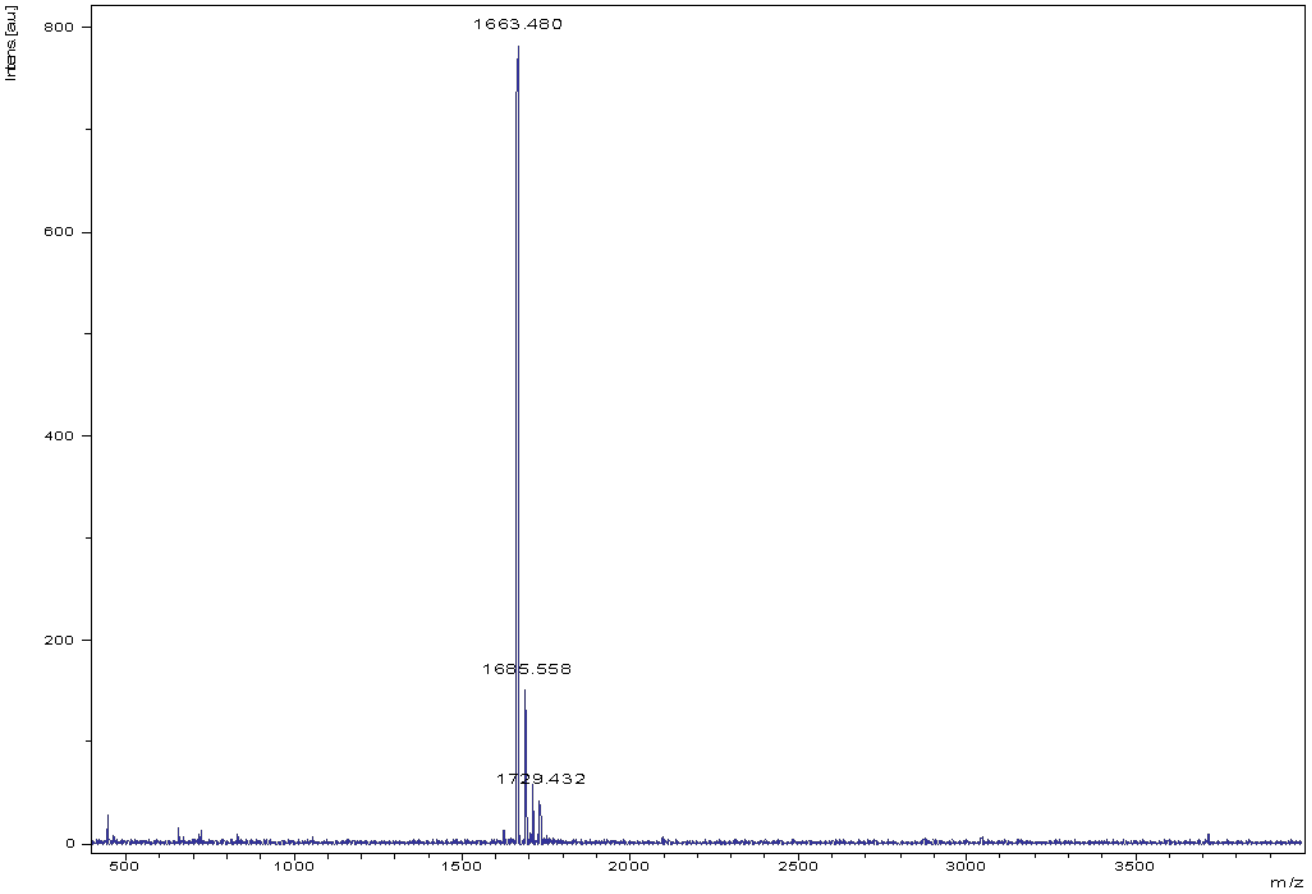
500
1000
1500
2000
2500
3000
3500
m/z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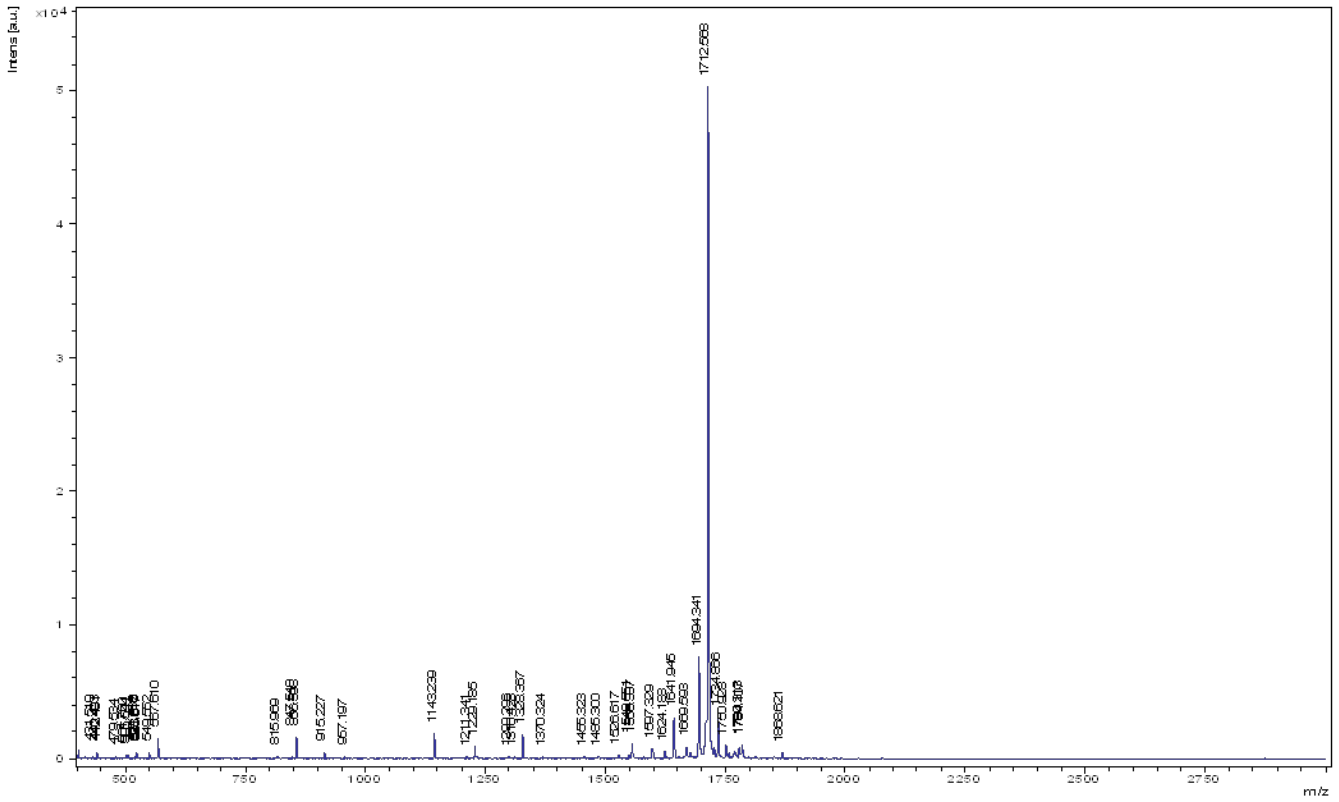
3000
3500
m/z


Intens [a.u.]
×10⁴
5
4
3
2
1
0
500
750
1000
1250
1500
1750
2000
2250
2500
2750
m/z
1712.558
1694.341
1143.239
915.227
957.197
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1455.323
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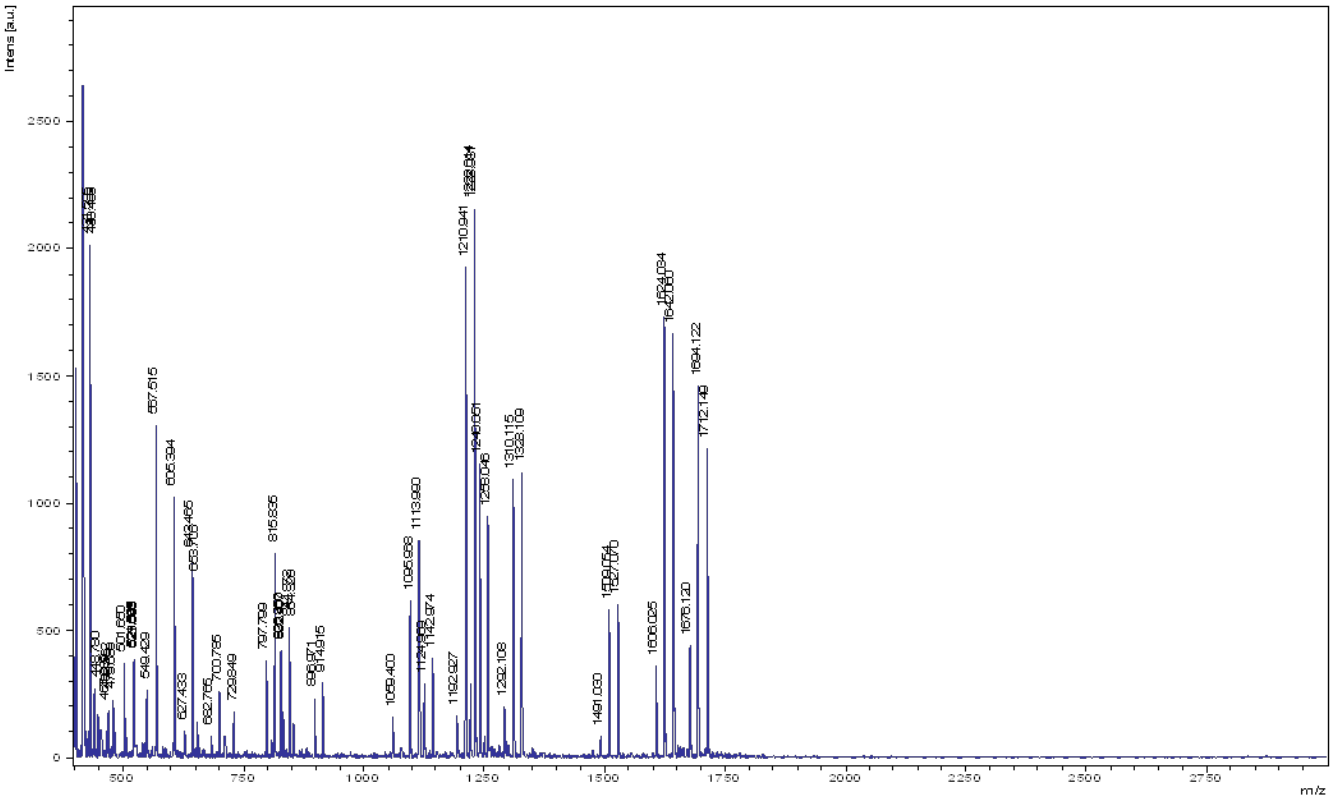
1669.583
1641.945
1624.183
1597.329
1868.621


Intens [a.u.]
2500
2000
1500
1000
500
0
500
750
1000
1250
1500
1750
2000
2250
2500
2750
m/z
557.515
606.394
627.433
662.766
700.795
729.849
797.799
815.835
914.915
1059.400
1095.988
1113.990
1142.974
1192.927
1210.941
1255.046
1292.108
1310.115
1491.030
1605.025
1624.094
1642.080
1676.120
1694.122
1712.143