Supporting Information

On-demand retrieval of cells three-dimensionally seeded in injectable thioester-based hydrogels

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Materials and Methods

Materials

Four-armed poly(ethylene glycol)s (M_w = 10,000) functionalized with sulfhydryl (Tetra-PEG-SH) and *N*-hydroxysuccinimide (Tetra-PEG-NHS) were purchased from NOF Co. Ltd. (Tokyo, Japan) and SINOPEG (Fujian, China), respectively. L-Cysteine methyl ester hydrochloride (L-cys), 4% paraformaldehyde phosphate buffer solution (PFA), 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sucrose were purchased from FUJIFILM Wako Chemicals Co. Ltd. (Osaka, Japan) and used without purification. Dulbecco's modified Eagle Medium (DMEM), penicillin-streptomycin (PS) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (MA, USA) and used without purification.

Hydrogel preparation

The thioester-based hydrogels were prepared from Tetra-PEG precursors at three different concentrations (20, 50, and 100 g/L). To prepare the hydrogels, Tetra-PEG-SH and Tetra-PEG-NHS were dissolved in HEPES buffer (200 mM, pH 8.2) and pure water, respectively. Equal amounts of these precursors were then mixed, and the mixture was poured into a mold. The samples were left for 24 h at ambient temperature to allow the completion of the gelation reaction before use in the experiments.

Rheological observation of hydrogels

To investigate the time-dependent change in the storage modulus (*G'*) and loss modulus (*G''*) after mixing Tetra-PEG precursors, rheological observation was performed using a rheometer (MCR301 and MCR302, Anton Paar, Austria). Briefly, 5 mL of the gelling solution was poured into a mold (CC17-SS), followed by immediate immersion of the concentric cylinder measurement head (CC17, diameter: 16.65 mm) into the solution. This procedure was performed with a shear amplitude γ = 1% and an oscillating frequency ω = 10 Hz at 25 °C. After the completion of the gelation reaction, *G*' and *G*" were continuously monitored at certain angular frequencies to determine the frequency dependence of the hydrogels. These measurements were conducted with a shear amplitude γ = 1% and oscillating frequency ω = 0.1–10 Hz at 25 °C.

FT-IR spectroscopy

The 100 g/L hydrogel was completely swollen and washed with excess deionized water before being lyophilized and ground for FT-IR measurements. FT-IR spectra of Tetra-PEG-HS, Tetra-

PEG-NHS, and the dried portion of the 100 g/L hydrogel were recorded using FT/IR-6300 (JASCO Corp., Japan) with a spectral resolution of 4 cm^{-1} (128 scans) and 1 cm^{-1} intervals.

Swelling and degradation behaviors

The swelling and degradation behaviors were characterized by microscopic observation of cylinder-shaped hydrogels (10 mm length, 1.9 mm diameter). The prepared hydrogels were immersed in 30 mL of phosphate buffer (100 mM, pH 6.8, 7.4, and 8.0) with L-cys at various concentrations (0, 1, 2, 5, and 10 mM). The swelling ratio (Q) was calculated from two values: as-prepared diameter (d_{ini}) and swelled diameter (d_{swe}) of hydrogels observed through an optical microscope (M165C, Leica); $Q = (d_{swe}/d_{ini})^3$.

Encapsulation of mesenchymal stem cells

Human mesenchymal stem cells (hMSCs, passage 2, 1.0×10^6 cells) were expanded and cultured on a 100 mm × 20 mm dish (Corning, USA) with 10 mL of DMEM containing 10% FBS and 1% PS at 37 °C under 5% CO_2 for a week. The medium was replaced with fresh medium every two or three days. The encapsulation of hMSCs into the hydrogel was performed using a protocol similar to that used for hydrogel preparation, except for the dispersion of hMSCs in the Tetra-PEG-SH. Briefly, hMSCs (1.0×10^5 cells) were dispersed in 10 μL of filter-sterilized HEPES buffer (200 mM, pH 8.2) with 100 g/L Tetra-PEG-SH, and the cell suspension was mixed with 10 μL of filter-sterilized water or 10% sucrose aq. with 100 g/L Tetra-PEG-NHS. This procedure was performed in a 1.5-mL sampling tube for 30 min at 37 °C under 5% CO_2 to allow cross-linking. The final cell density in the hydrogels was 5.0×10^6 cells/mL.

Confocal laser scanning microscopy (CLSM) observation of hMSCs encapsulated in hydrogels

To evaluate the three-dimensional (3D) distribution and cytocompatibility, hMSCs encapsulated in hydrogels were visualized using CLSM (Zeiss, Germany). Briefly, hMSCs were stained with 500 μL of DMEM with 1 μM calcein-AM (live cells; green) and 2 μM ethidium homodimer (dead cells; red) for 10 min, and then were fixed with 500 μL of 4% PFA for 10 min. This procedure was performed at 37 °C under 5% CO_2 in the dark, and washing with PBS was performed three times to ensure the diffusion of the molecules in and out of the specimens. The 3D distribution images of the hMSCs in the hydrogels were obtained by stacking sequential images sliced with a thickness of 2.98 μm for the 150 μm of z-dimension. To evaluate cytocompatibility after

encapsulating the hMSCs into the hydrogels, the total numbers of live and dead cells were counted using ImageJ software.

Cell viability after being exposed to L-cys solution

To evaluate the cell viability during degradation, 200 μL of phosphate buffer (100 mM, pH 7.4) with L-cys (0, 1, 2, 5, and 10 mM) was mixed with hMSCs (final cell density: 5.0×10^6 cells/mL). After exposure for 30 and 60 min, the cells were stained with trypan blue, and cell viability was evaluated using a cell counter (Thermo Fisher Scientific).

Hydrogel degradation, cell retrieval, and two-dimensional culture

After encapsulating hMSCs into hydrogels, the hydrogels were degraded with phosphate buffer (100 mM, pH 7.4) with 5 mM L-cys. To complete the degradation of the hydrogels, they were left 1 h at 37 °C under 5% CO_2, followed by seeding the retrieved hMSCs on 48-well culture plates at a cell density of 3.0×10^4 cells/well. After 2, 5, and 7 days, the viability, cell number, and mitochondrial activity were determined using the following procedures:

For the cell viability assay, hMSCs were stained with calcein-AM and ethidium homodimer, as described above. Fluorescence images of hMSCs were obtained and analyzed using fluorescent microscopy.

For cell number analysis, adhered hMSCs were detached by adding 300 μL of 1% trypsin-EDTA and incubated for 5 min at 37 °C under 5% CO_2. The detached cells were collected and centrifuged for 5 min at 1,000 rpm. After removing the supernatant, 200 μL of DMEM was added, and the cell number was determined using a cell counter, as described above.

For mitochondrial analysis, MTT was used. hMSCs were incubated with DMEM with 0.5 mg/mL MTT at 37 °C under 5% CO_2 for 4 h. The solution was removed, and the plate was washed three times with PBS. To ensure the diffusion of purple formazan salts, isopropanol with 0.04 M HCl was added, followed by incubation overnight. Finally, the absorbance of the resulting solution at 570 nm was measured using a Nano Drop spectrophotometer (Thermo Fisher Scientific).

Statistical analysis

Three samples were tested to ensure the statistical reliability. The differences between the mean values of the control and sample groups were calculated to assess the statistical significance.

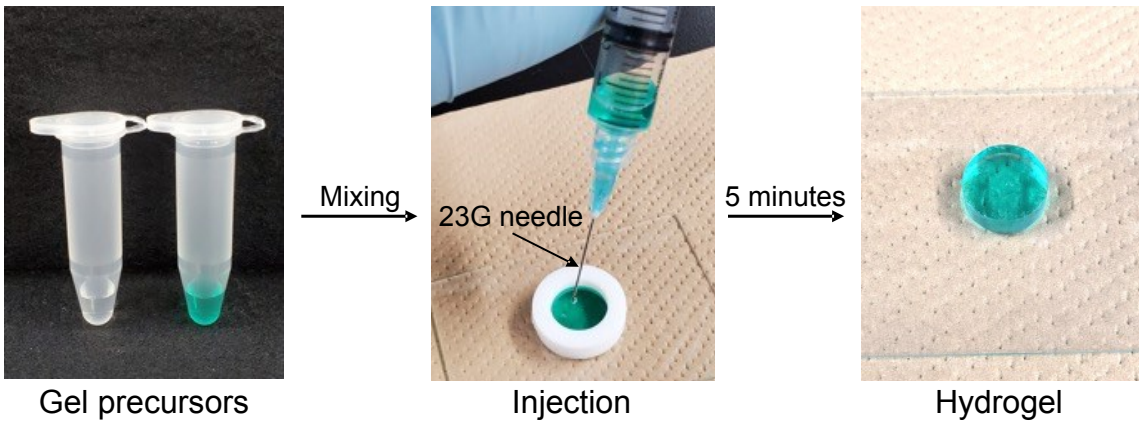

Figure S1. Formation process of the 100 g/L hydrogel via a syringe. PEG-SH was stained with a blue ink (1 vol%).

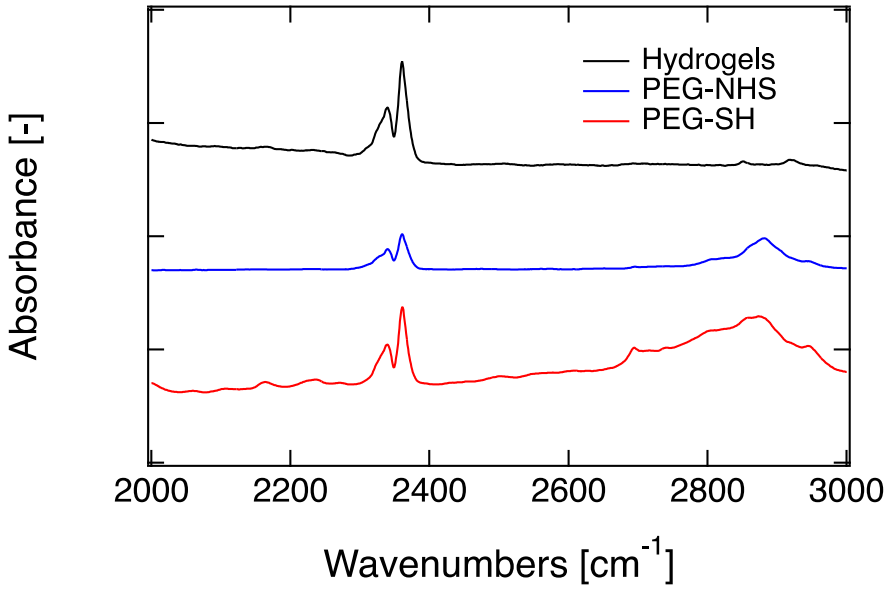

Figure S2. FT-IR spectra of Tetra-PEG-HS, Tetra-PEG-NHS, the dried portion of the 100 g/L hydrogel.

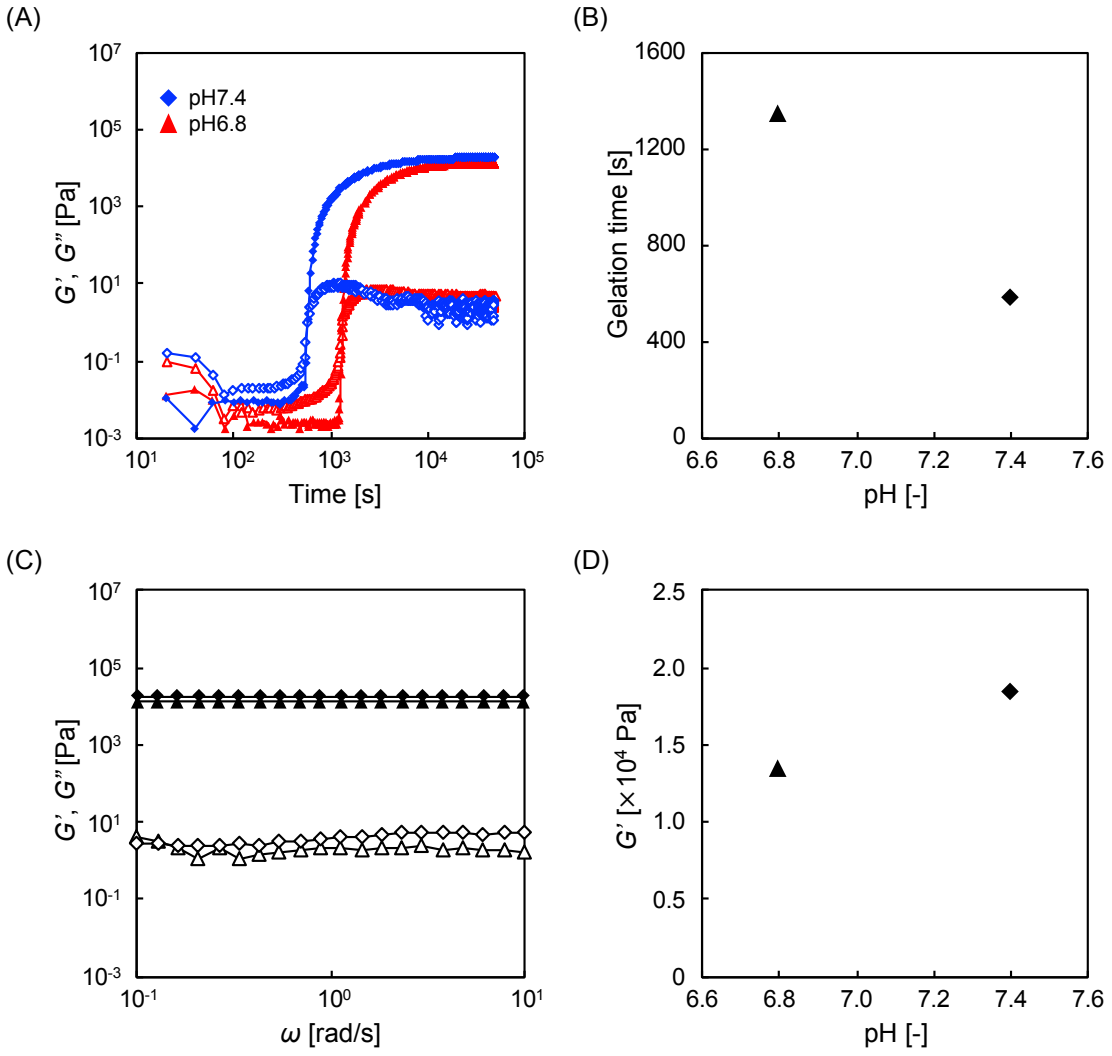

Figure S3. Rheological properties of hydrogels prepared at pH 6.8 and pH 7.4. (A) Observation of *G*' (closed symbols) and *G*'' (open symbols). For readability, the data points after 2,000 s were reduced. (B) Gelation time as a function of pH. (C) Angular frequency dependence of *G'* and *G''*. (D) *G'* obtained at 10 rad/s as a function of pH.

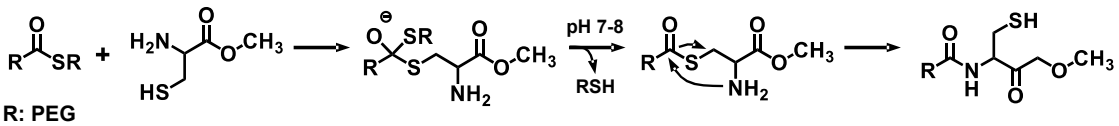

Figure S4. Thiol-thioester exchange reaction.

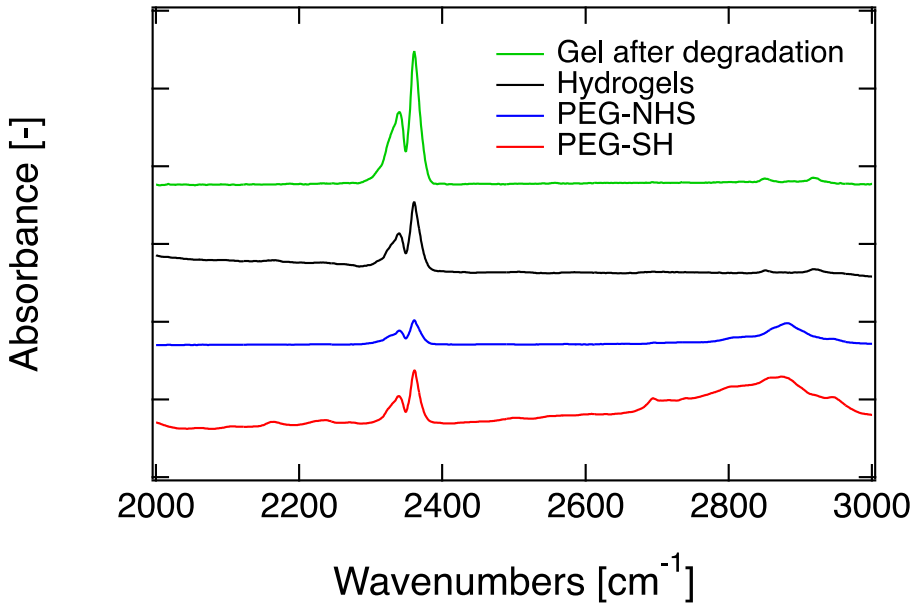

Figure S5. FT-IR spectra of PEG-HS, PEG-NHS, the dried portion of the 100 g/L hydrogel, and the dissolved hydrogel.

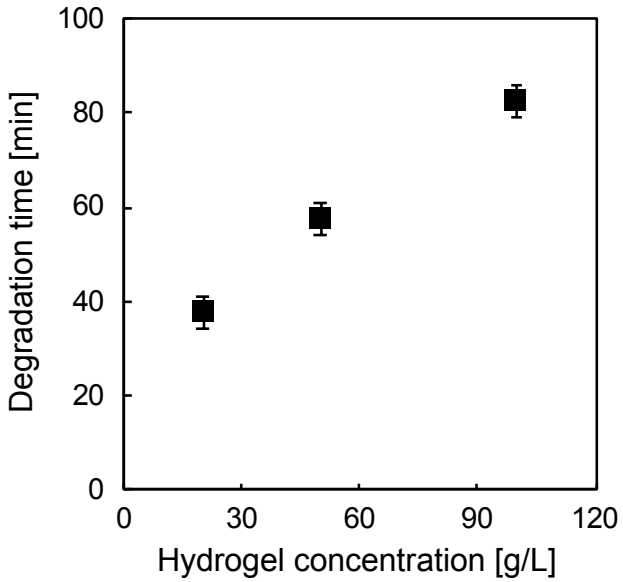

Figure S6. Degradation time of hydrogels stimulated by 5 mM L-cys at 25 °C.

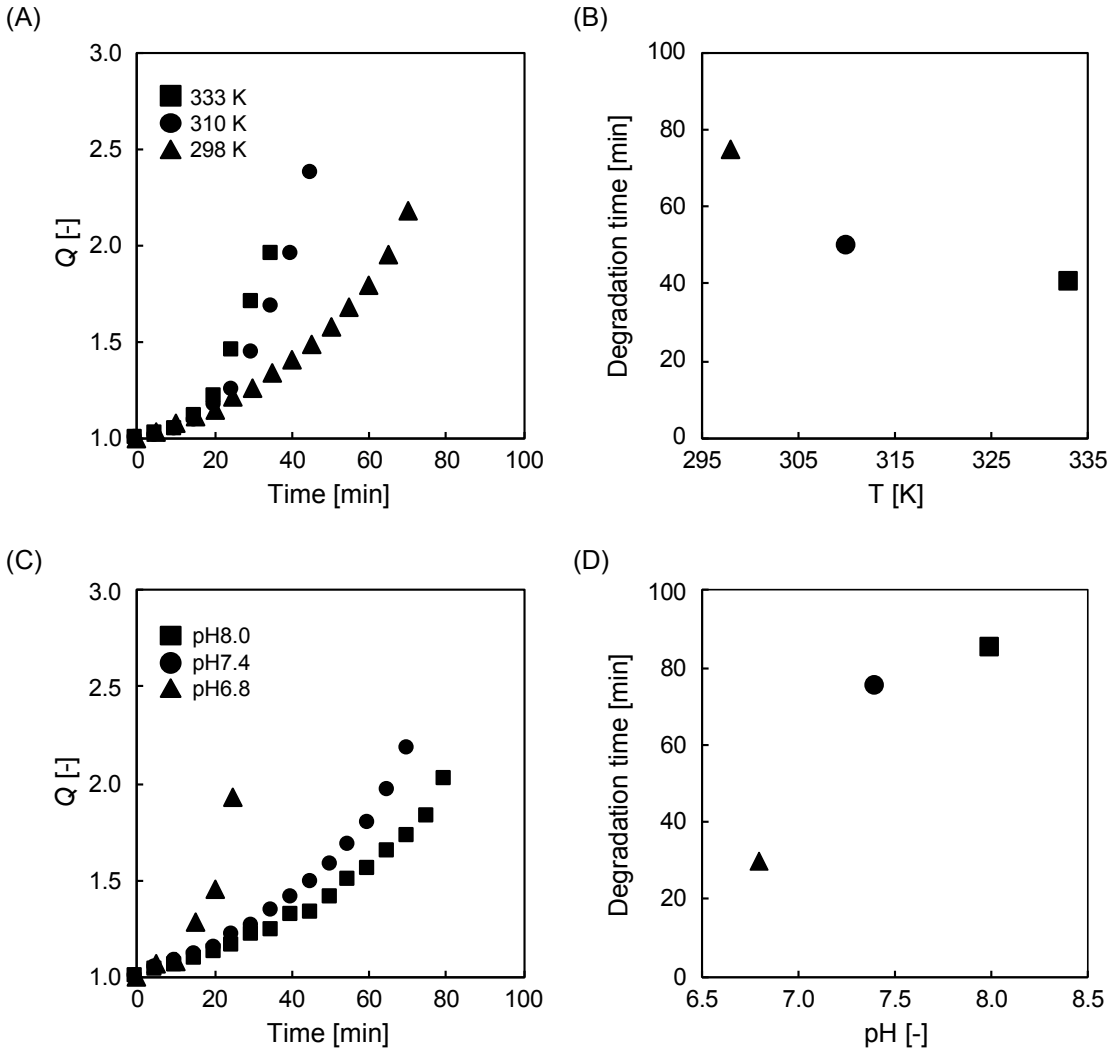

Figure S7. Degradation profile of 100 g/L hydrogel stimulated by 5 mM L-cys under various conditions. (A) Temperature dependence of *Q*. (B) Degradation time at various temperatures. (C) pH dependence of *Q*. (D) Degradation time under various pH conditions.

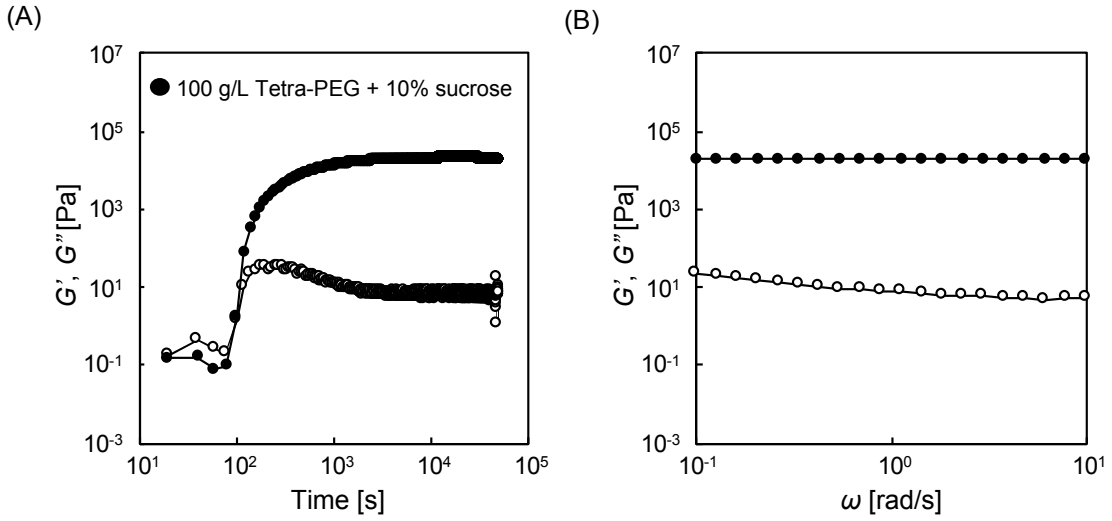

Figure S8. Rheological properties of hydrogels mixed with 10% sucrose. (A) Observation of *G*' (closed symbols) and *G*'' (open symbols). (B) Angular frequency dependence of *G*' and *G*''.

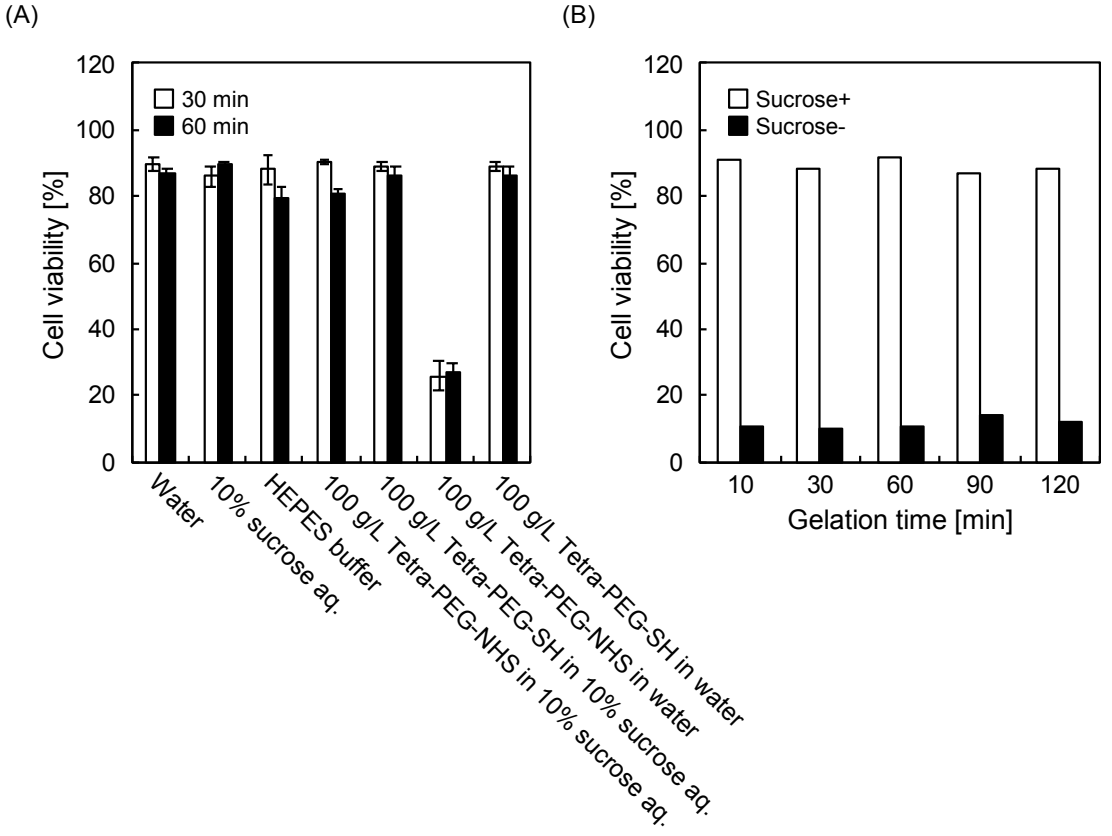

Figure S9. Cell viability in various conditions. (A) Cell viability of hMSCs suspended in various solutions. (B) Cell viability as a function of gelation time for hMSCs encapsulated in hydrogels.

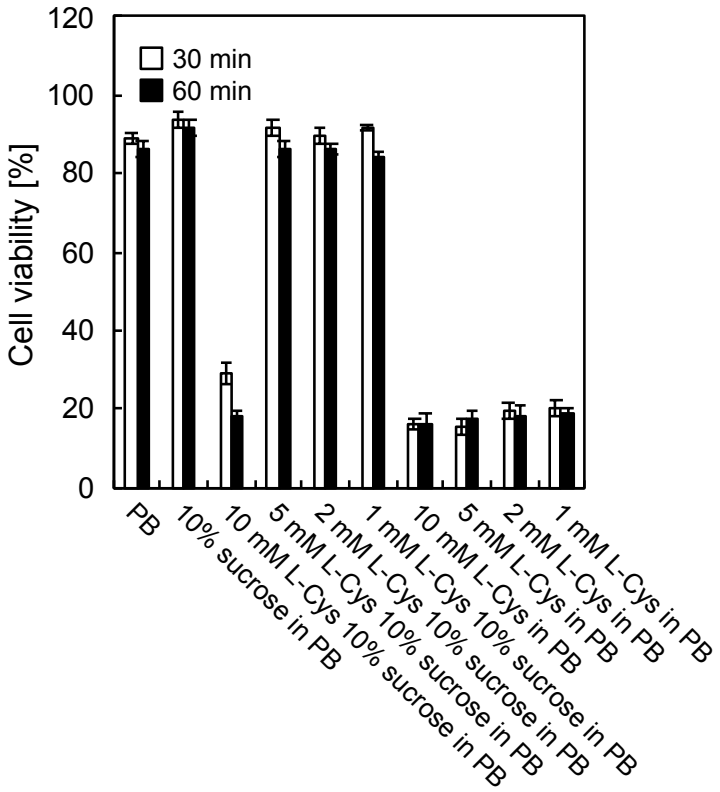

Figure S10. Cell viability in various conditions. PB: phosphate buffer.