Enzyme-mediated preparation of hydrogels composed of poly(ethylene glycol) and gelatin as cell culture platforms

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Materials

Thiol-functionalized 4-arm poly(ethylene glycol) (4-arm PEG-SH, M_W: 20000) was purchased from NOF CORPORATION (Tokyo, Japan). Horseradish peroxidase (HRP, 100 units/mg) and dithiothreitol (DTT) were purchased from Wako Chemicals (Osaka, Japan). Tyramine hydrochloride, cystamine, sodium azide and gelatin from porcine skin were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-cysteine (Cys) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) and Cellstain-double staining kit were purchased from Dojindo (Kumamoto, Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from the Peptide institute (Osaka, Japan). The center of a 25-mm diameter cell culture dish (Cat. No. 3815-012) was purchased from Iwaki Glass (Tokyo, Japan). Mouse fibroblast L929 cells (RCB1451) were provided by the Riken Cell Bank (Tsukuba, Japan).

Methods

Synthesis of thiolated gelatin (Gela-SH)

Gelatin (2.0 g) was dissolved in 100 ml of MilliQ water. Cystamine (1.0 g, 4.5 mmol) was then added with stirring. The pH of the mixture solution was adjusted to 4.75 by addition of 1.0 M HCl. Next, EDC (0.86 g, 4.5 mmol) was added in solid form. The pH of the mixture solution was maintained at 4.75 by the addition of 1.0 M HCl. After 2 h of stirring at room temperature, the reaction was arrested by the addition of 1.0 M NaOH to increase the pH to 7.0. DTT (8.5 g) was added and the pH of the mixture solution was further increased to 8.5 by the addition of 1.0 M NaOH. After 1 day of stirring, the pH of the mixture solution was adjusted to 3.5 by the addition of 1.0 M HCl to avoid self-crosslinking of the thiol moieties of Gela-SH. The mixture solution was transferred to a dialysis tube (MWCO 10,000) and dialyzed exhaustively against HCl solution (pH 3.5) for 3 days. The mixture solution was then lyophilized to yield Gela-SH. The degree of substitution (DS) of Gela-SH was evaluated by Ellman's test[1]. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was dissolved in 100 mM Tris(hydroxymethyl)aminomethane-HCl (Tris-HCI) buffer (pH 8.0). Gela-SH was dissolved in phosphate-buffered saline (PBS) at 1 mg/mL. Gela-SH solution (40 μL) was then mixed with DTNB solution (160 μL). After 2 h of stirring under darkened conditions at room temperature, the absorbance was measured with a microplate reader (Power Wave X, Bio-Tec Instruments Inc., USA) at a wavelength of 412 nm. The absorbance of the Gela-SH solution (1 mg/mL) was 0.58. A calibration curve was obtained by measuring the absorbance of known concentrations of cysteine solutions (Figure S1). DS of Gela-SH was calculated from the calibration curve ([Abs] = 1.29 × [SH]) with the absorbance of Gela-SH solution at 412

nm (0.58), which resulted in c.a. 0.45 mmol-SH/g-gelatin.

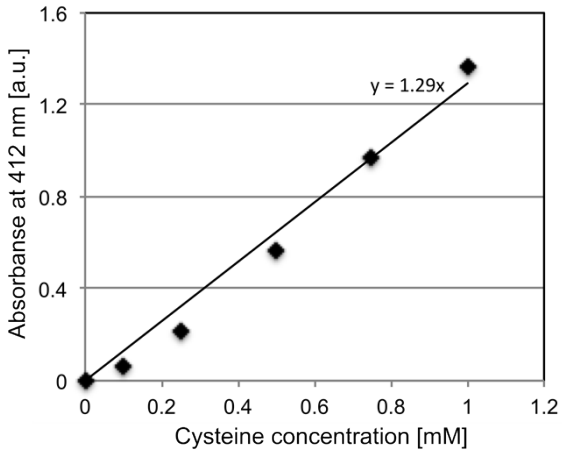

Figure S1. A calibration curve obtained by measuring the absorbance of cysteine solution.

Preparation of (PEG-SH)-(Gela-SH) hydrogel

PEG-SH and Gela-SH were dissolved in PBS (pH 7.4). Each polymer solution, HRP solution (30 U/mL) and tyramine solution (30 mM) were mixed at a volume ratio of 2 : 2 : 1 : 1 (PEG-SH : Gela-SH : HRP : tyramine). The mixture solution was allowed to stand still at room temperature to yield (PEG-SH)-(Gela-SH) hydrogels. The final concentrations of PEG-SH and Gela-SH in the hydrogel formation are shown in Table 1.

Equilibrium swelling ratio (Q_M) and gel content

(PEG-SH)-(Gela-SH) hydrogels (300 μL) in the shape of a disk (~1.5 cm in diameter and ~2 mm in thickness) were incubated in 10 mL of PBS (pH 7.4) containing 0.1% w/v sodium azide at 37 °C for 4 days to reach equilibrium. Their mass after swelling (M_S) was measured. The hydrogels were then dried, and their dry mass (M_D) was measured. The equilibrium swelling ratio (Q_M) was calculated according to the equation, $Q_M = M_S / M_D$.

The gel content of the (PEG-SH)-(Gela-SH) hydrogels was determined as follows. Samples of polymer (polymer mass: W_p) were converted into hydrogels in the shape of a disk ~1.5 cm in diameter and ~2 mm as described above. The hydrogels were subsequently incubated in 10 ml of MilliQ water and the solution was refreshed four times for 4 days to remove salts and uncross-linked polymers. The hydrogels were then dried, and their dry mass (W_D) was measured. The gel content was calculated according to the equation, gel content = $W_D / W_p \times 100\%$.

Rheological experiment

Rheological evaluation of (PEG-SH)-(Gela-SH) hydrogels was carried out with a MCR302 rheometer (Anton Paar) using parallel plate (50 mm). Storage modulus (*G'*) was measured as a function of frequency from 0.1 to 10 Hz. The measurement was performed through the oscillation frequency sweep test at room temperature (25 °C), while the strain was set at 1%..

Adhesion and proliferation of cells

Cellular adhesiveness and proliferation on the (PEG-SH)-(Gela-SH) hydrogel were evaluated using mouse fibroblast L929 cells. (PEG-SH)-(Gela-SH) hydrogel sheets (500 μL) were prepared in the center of a 25-mm diameter cell culture dish. After hydrogelation, gel sheets were rinsed using PBS and minimum essential medium (MEM) containing 10% fetal bovine serum (FBS). After washing, L929 cells suspended in MEM containing 10% FBS were seeded onto the hydrogel sheets at 2×10^5 cells/mL and 2×10^4 cells/mL to evaluate the adhesiveness of cells after 4 h of seeding and their growth profiles for 1 week, respectively. After an appropriate incubation, L929 cells were recovered by treatment with trypsin to determine the number of adherent cells. A hydrogel sheet prepared from 5% (w/v) PEG-SH and 0.1% (w/v) native gelatin was employed in a negative control experiment. Cellular adhesiveness on the (PEG-SH)-(native gelatin) hydrogel was evaluated in the same manner as mentioned above.

A gelatin-coated cell culture dish as a positive control was prepared as follows. Native gelatin solution (0.1% (w/v)) was poured into each well. After incubation for 1 h, each well was washed with PBS. The cell culture dish was used as a gelatin-coated dish.

Harvesting cell sheets

L929 cells were cultured until a confluent monolayer formed on the P5G0.1 hybrid hydrogel sheets in the center of a 25-mm diameter cell culture dish. After cultivation, the cell culture medium was removed and 10 mM Cys solution (5 mL) was poured into each well. After incubation at 37 °C for 30 min, the cell sheet was transferred to a fresh cell culture dish to evaluate their re-attachment onto other surfaces. After 24 h of cultivation, the cell sheet that was re-seeded on the cell culture dish was stained with a fluorescein double-staining kit.

References

[1] G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, 82, 70

Table S1. Total thiol concentration of each condition.

samples	Total thiol concentration [mM]
P5G0	10
P5G0.01	10.045
P5G0.1	10.45

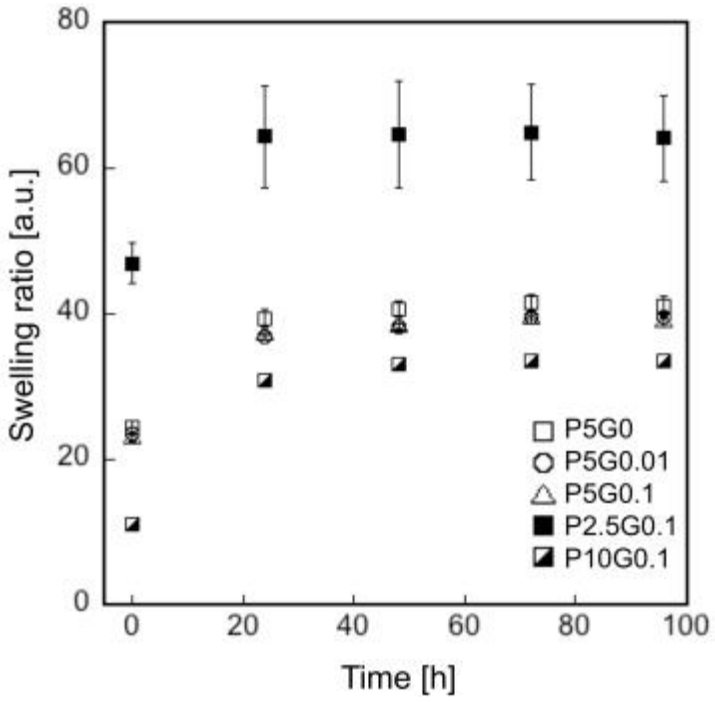

Figure S2. Swelling ratio of (PEG-SH)-(Gela-SH) hybrid hydrogels as a function of time. Error bars denote standard deviations (SD) (n = 3).

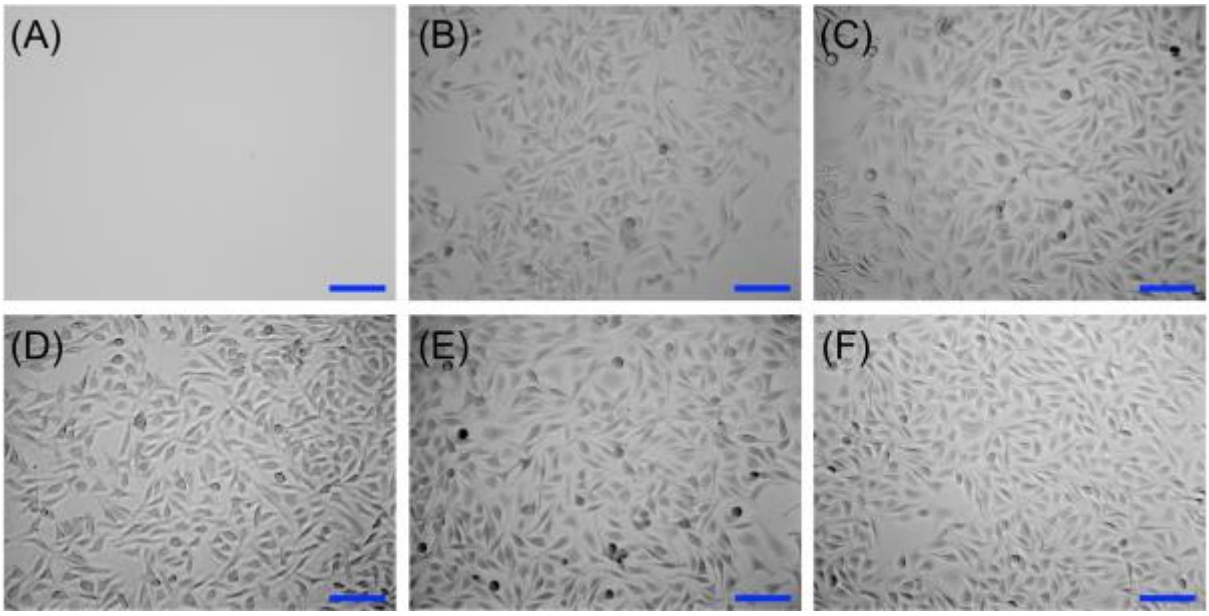

Figure S3. Photomicrographs of L929 cells on (A) P5G0, (B) P5G0.01, (C) P5G0.1, (D) P2.5G0.1, (E) P10G0.1 and (F) gelatin coat dish, respectively, at 4 h of cell seeding. Bars: 100 μm.

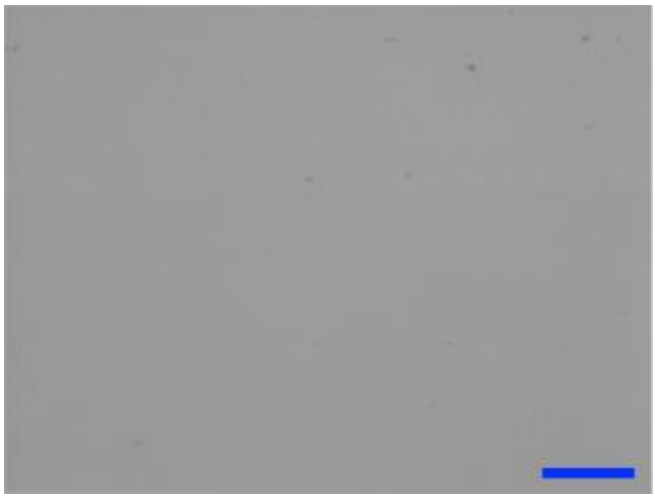

Figure S4. Photomicrographs of L929 cells on hydrogel prepared from 5% (w/v) PEG-SH and 0.1% (w/v) native gelatin at 4 h of cell seeding. Bars: 100 μm.

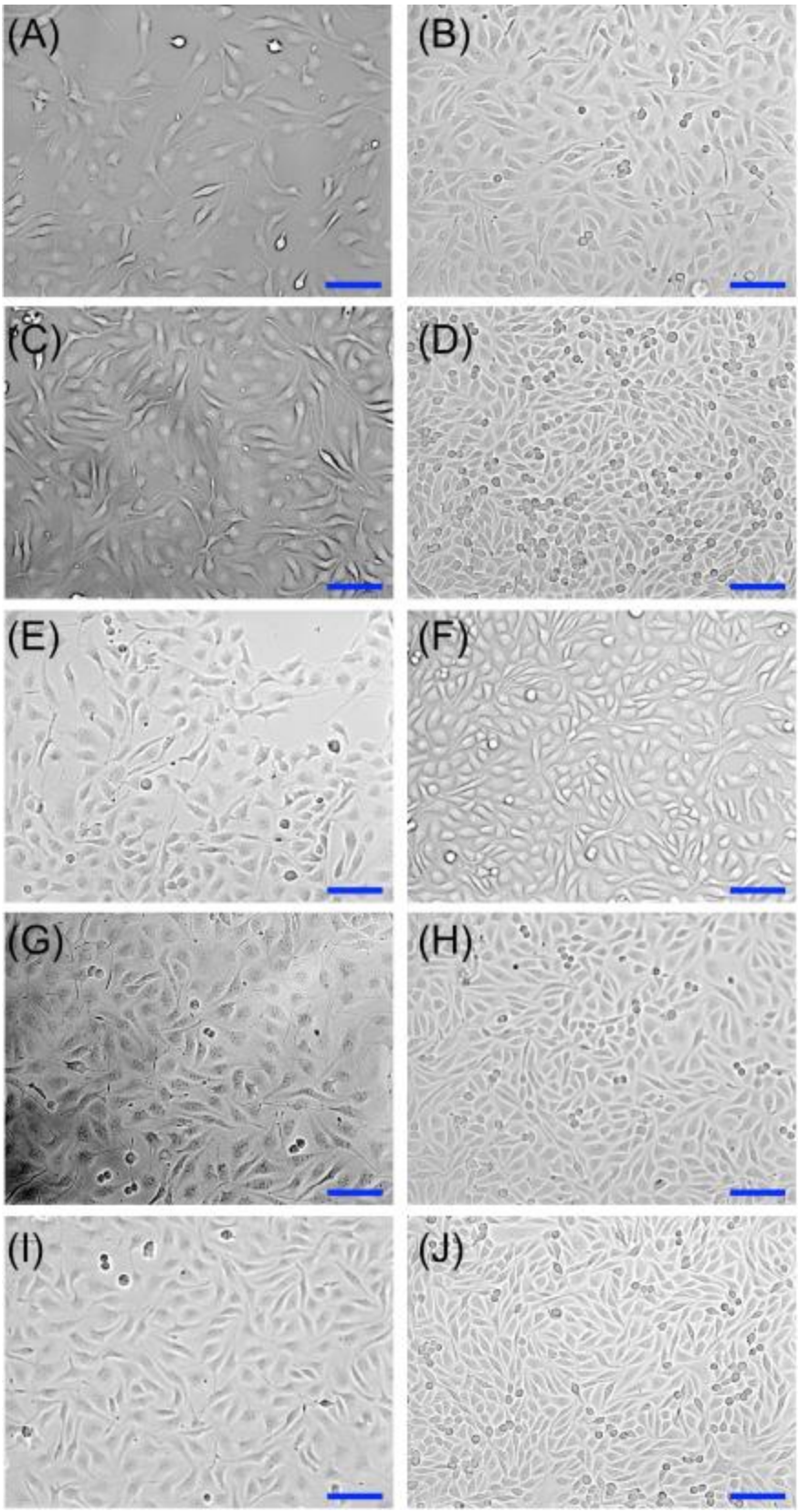

Figure S5. Photomicrographs of L929 cells on (A, B) P5G0.01, (C, D) P5G0.1, (E, F) P2.5G0.1, (G, H) P10G0.1 and (I, J) gelatin coat dish, respectively, after (A, C, E, G, I) 3 d and (B, D, F, H, J) 5 d of cultivation. Bars: 100 μm.

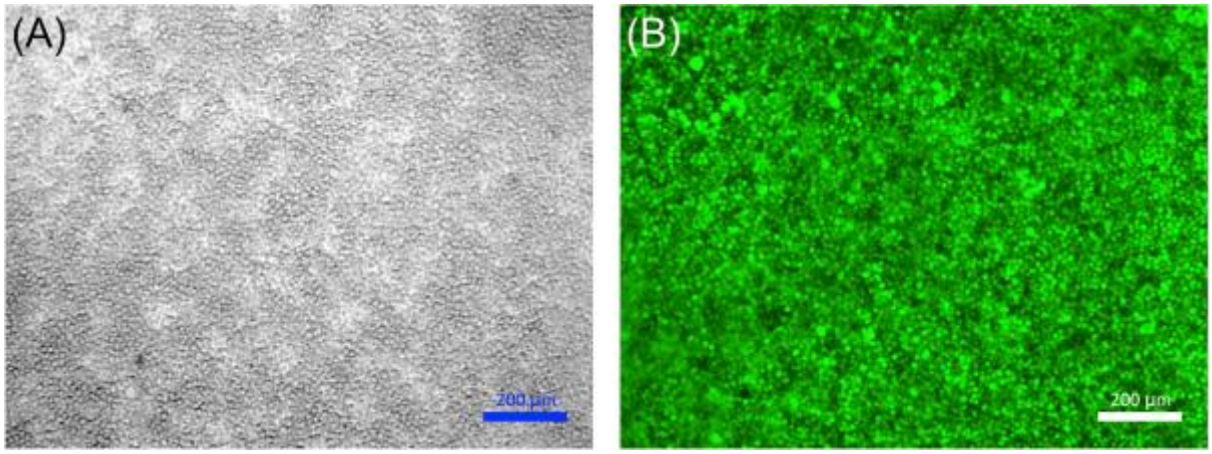

Figure S6. Photomicrographs of L929 cell sheet after 24 h of re-seeding on a fresh cell culture dish. (A) Bright field image and (B) fluorescence image, respectively. Bars: 200 µm.