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Enzymatic preparation of a redox-responsive hydrogel for encapsulating and releasing living cells

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Materials

Thiol-functionalized 4-arm poly(ethylene glycol) (4-arm PEG-SH, M_W: 20,000) was purchased from NOF CORPORATION (Tokyo, Japan). Horseradish peroxidase (HRP, 100 units/mg), resorcinol, dithiothreitol (DTT), and catalase were purchased from Wako Chemicals (Osaka, Japan). Tyramine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenol, glycyl-L-tyrosine hydrate (Gly-Tyr), serotonin hydrochloride, and L-cysteine (Cys) were 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). Mouse fibroblast L929 cells (RCB1451) were provided by the Riken Cell Bank (Tsukuba, Japan).

Methods

Gelation test of 4-arm PEG-SH solution

4-arm PEG-SH hydrogel (300 μL) was prepared in a 10-mm diameter glass vessel at room temperature. 4-arm PEG-SH was dissolved in phosphate-buffered saline (PBS, pH 7.4) at 10 wt.%. PBS solutions of HRP (75 μL, 20 U/mL) and phenolic compound (75 μL, 20 mM) were added to the polymer solution (150 μL), during stirring by gentle pipetting. The final concentrations of 4-arm PEG-SH, HRP, and phenolic compounds were 5 wt.%, 5 U/mL, and 5 mM, respectively.

Effect of catalase on the hydrogelation of 4-arm PEG-SH solution

4-arm PEG-SH was dissolved in PBS (pH 7.4) at 15 wt.%. Fifty microliters of HRP (30 U/mL), and 100 μL of tyramine hydrochloride (15 mM), in PBS, were added to the polymer solution (100 μL). Catalase solution (50 μL, 263.2 μg/mL) was subsequently added with stirring by gentle pipetting. The final concentrations of 4-arm PEG-SH, HRP, tyramine hydrochloride, and catalase were 5 wt.%, 5 U/mL, 5 mM, and 43.9 μg/mL, respectively.

Degradation of 4-arm PEG-SH hydrogels with dithiothreitol (DTT)

4-arm PEG-SH hydrogel was prepared in a glass vessel as described above (final concentrations of 4-arm PEG-SH, HRP, and phenolic compounds were 5 wt.%, 5 U/mL, and 5 mM, respectively). DTT solution (500 μL) was poured into the glass vessel, which was then incubated for 1 h at 37°C.

Ellman's test

4-arm PEG-SH hydrogel (100 μL) was prepared in a glass vessel as described previously (final concentrations of 4-arm PEG-SH, HRP, and tyramine hydrochloride were 5 wt.%, 5 U/mL, and 5 mM, respectively). After standing for 1 day, 1000 μL of sodium azide solution (100 mg/mL) was poured into the glass vessel, which was then incubated for >24 h to inactivate the HRP. Five hundred microliters of DTNB solution (10 mM) was then poured into the glass vessel. After 2 h of incubation, the absorbance of the supernatant was measured at 412 nm.

4-arm PEG-SH hydrogel was subsequently degraded by DTT solution (25 mM). The solution was dialyzed against deionized water for 2 days to remove residual DTT. The decomposed product was obtained by freeze-drying for 2 days. The decomposed product was dissolved in PBS, and the thiol group content of the solution was evaluated as mentioned above.

Gelation time measurement

The gelation time of the 4-arm PEG-SH aqueous solution was determined according to previous reports.[1, 2] 4-arm PEG-SH was dissolved in PBS (pH 7.4) at 10 wt.%. The polymer solution was placed into a 48-well plate, with 100 μL/well. Fifty microliters of HRP (20 U/mL) in PBS was then poured into each well, and stirred at 200 rpm using magnetic stirrer bars (length, 7 mm; width, 3 mm) for 10 s. Fifty microliters of phenolic compound (20 mM) was then finally poured into the wells, under stirring at room temperature. The final concentrations of 4-arm PEG-SH, HRP, and phenolic compound were 5 wt.%, 5 U/mL, and 5 mM, respectively. Gel formation was deemed to have occurred when the magnetic stirrer bar was hindered and the surface of the mixture solution appeared swollen. Gelation time was measured at different concentrations of polymer, HRP and tyramine hydrochloride.

Rheological properties measurement

Rheological evaluation of the hydrogels was carried out with MCR302 rheometer (Anton Paar) using cone plate (25 mm diameter, 2.003°). 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), and the strain was set at 1%. The final concentrations of 4-arm PEG-SH, HRP and tyramine hydrochloride were, 5, 10 and 15 wt%, 5 U/ml and 5 mM, respectively.

Equilibrium swelling (Q_M) ratio and gel content

4-arm PEG-SH hydrogels were prepared as described above (final concentrations of 4-arm PEG-SH, HRP, and tyramine hydrochloride were 5, 10 and 15 wt%, 5 U/mL, and 5 mM, respectively). After standing for 4 h at room temperature, 4-arm PEG-SH hydrogel, in the shape of a disk ~1.5 cm in diameter and ~2 mm in thickness, was incubated in 10 ml of PBS (pH 7.4) at 37°C for 3 days to reach equilibrium. Their mass after swelling (M_S) was measured. The hydrogels were then dried in an oven, 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 4-arm PEG-SH hydrogels was determined as follows. Samples of 15, 30 and 45 mg (W_p) of 4-arm PEG-SH were converted into hydrogels (final concentrations of 4-arm PEG-SH, HRP and tyramine hydrochloride were 5, 10 and 15 wt%, 5 U/ml and 5 mM, respectively) in the sharp of a disk ~1.5 cm in diameter and ~2 mm. The hydrogels were subsequently incubated in 10 ml of MilliQ water and the solution was refreshed 4 times for 3 days to remove salts and uncross-linked polymer. The hydrogels were then dried in an oven, and their dry mass (W_D) was measured. The gel content was calculated according to the equation, gel content = $W_D / W_p \times 100\%$.

Fluorometric measurement of di-tyramine cross-linking formation

In this experiment, the reduced form of glutathione (GSH) was used instead of 4-arm PEG-SH. GSH was dissolved in PBS (pH 7.4) at 20 mM. PBS solutions of HRP (125 μL, 20–200 U/mL) and tyramine hydrochloride (125 μL, 4 mM) were added to the GSH solution (250 μL). The final concentrations of GSH, HRP and tyramine hydrochloride were 10 mM, 5–50 U/mL, and 1 mM, respectively. The mixture was poured into a quartz cell, and the di-tyramine fluorescence was measured using a Perkin Elmer LS55 fluorometer, after appropriate incubation at room temperature. Fluorescence spectra at 350–500 nm were obtained upon excitation at 283 nm.

Degradation of 4-arm PEG-SH hydrogel with Cys

4-arm PEG-SH hydrogels were prepared as described previously (final concentrations of 4-arm PEG-SH, HRP, and tyramine hydrochloride were 5 wt.%, 5 U/mL, and 5 mM, respectively). After standing for hydrogelation at room temperature for 1 hour, 4-arm PEG-SH hydrogel, in the shape of a disk ~1.5 cm in diameter and ~2 mm in thickness, was submerged in PBS (10 mL) for 3 days to reach the equilibrium swelling. After that, all the hydrogels were soaked in PBS (5 ml) containing 0, 1, 5, or 10 mM Cys. Changes in hydrogel

weight were measured after appropriate incubation at 37 °C. The Cys solution was refreshed after 1 h of incubation.

Cell encapsulation and degradation of the hydrogel

An aqueous solution containing 4-arm PEG-SH, HRP, and L929 fibroblasts was mixed with tyramine solution. Five hundred microliters of mixed solution was placed into the center of a 35-mm diameter cell culture dish. The final concentrations of 4-arm PEG-SH, HRP, tyramine, and L929 cells were 5 wt.%, 5 U/mL, 5 mM, and 4×10^5 cells/mL, respectively. The mixture was incubated at 37 °C for 1 h. After gelation, minimum essential medium containing 10% fetal bovine serum was poured into the well. Cells encapsulated within the hydrogel were cultured for 3 and 24 h, under standard culture conditions (37 °C and 5% CO_2). After cultivation, live and dead cells within the hydrogel were stained using a fluorescence double staining kit. Six fluorescence microscopic fields (×40), each containing >100 cells per field, were randomly selected in three separate experiments. The number of living (stained green) and dead (stained red) cells was counted to estimate the viability of encapsulated cells within the hydrogel. To recover encapsulated cells, 5 mM Cys solution (5 mL) was poured into the well. After incubation at 37 °C for 30 min, recovered cells were seeded on the center of a 15-mm diameter cell culture dish. The morphology of cells was observed by optical microscopy after 4 and 48 h of cultivation. The viability of recovered cells was determined by trypan blue exclusion, using an automated cell counter (Bio-Rad Laboratories, Inc.).

References

[1] S. Sakai, K. Hirose, K. Taguchi, Y. Ogushi, K. Kawakami, *Biomaterials*, 2009, 30, 3371

[2] Y. Ogushi, S. Sakai, K. Kawakami, *J. Biosci. Bioeng.*, 2007, 104, 30

Table S1. Gelation time of 4-arm PEG-SH solution in the presence of various phenolic compounds. Data are presented as mean ± standard deviation (n = 3-6).

Phenol derivatives	Gelation time [h]
Tyramine	0.50 ± 0.02
Phenol	0.66 ± 0.07
Gly-Tyr	0.67 ± 0.08
Resorcinol	1.1 ± 0.05
Serotonin	8.7 ± 0.11

Table S2. Effect of 4-arm PEG-SH concentration on gelation time of polymer solution at pH 7.4. Data are presented as mean ± standard deviation (n = 3).

4-arm PEG-SH concentration [wt%]	Gelation time [min]
5	30.1 ± 0.9
10	30.0 ± 1.7
15	30.6 ± 2.0

Table S3. Effect of 4-arm PEG-SH concentration on equilibrium swelling ratio (Q_M) and gel content. Data are presented as mean ± standard deviation (n = 3).

4-arm PEG-SH concentration [wt%]	Equilibrium swelling ratio (Q_M) [-]	Gel content [%]
5	42.5 ± 0.4	85.4 ± 5.1
10	34.4 ± 0.7	83.5 ± 3.6
15	29.7 ± 0.7	81.1 ± 2.5

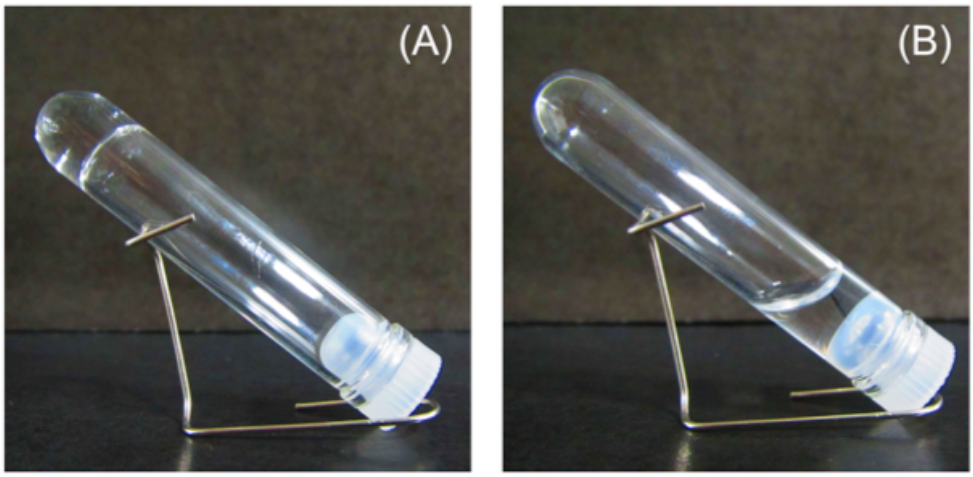

Fig. S1. Photographs of solutions containing PEG-SH, HRP, and tyramine in the (A) absence and (B) presence of catalase, at pH 7.4.

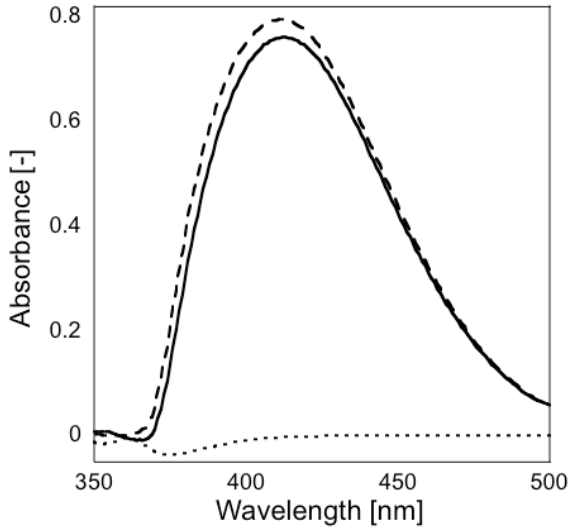

Fig. S2. Ultraviolet-visible absorption spectra of 4-arm PEG-SH solution (solid) before and (dotted) after hydrogelation, and (dashed) the decomposed hydrogel using Ellman's method.

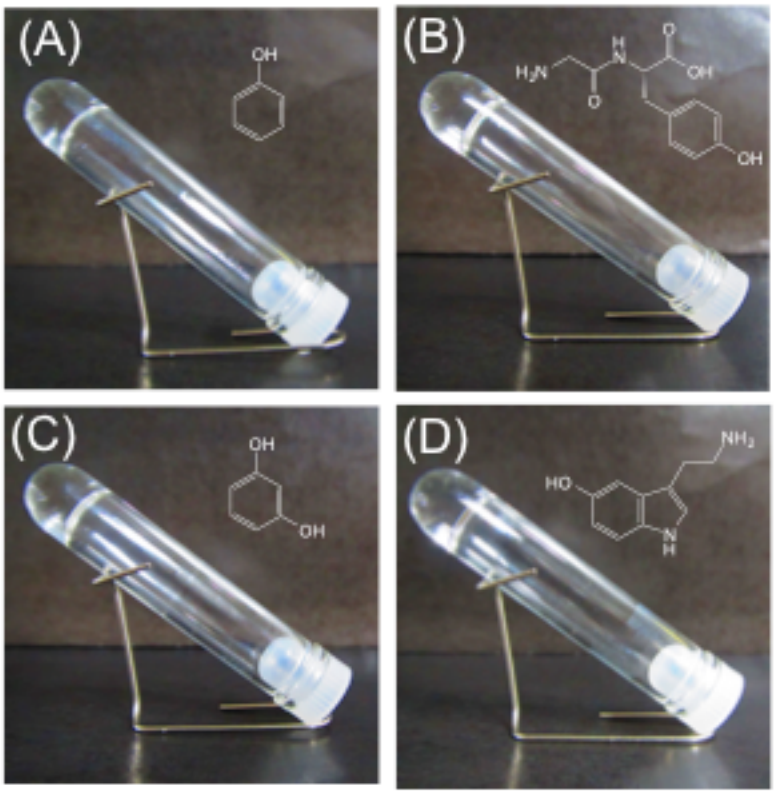

Fig. S3. Photographs of solutions containing PEG-SH and HRP with the addition of (A) phenol, (B) Gly-Tyr, (C) resorcinol, and (D) serotonin, at pH 7.4.

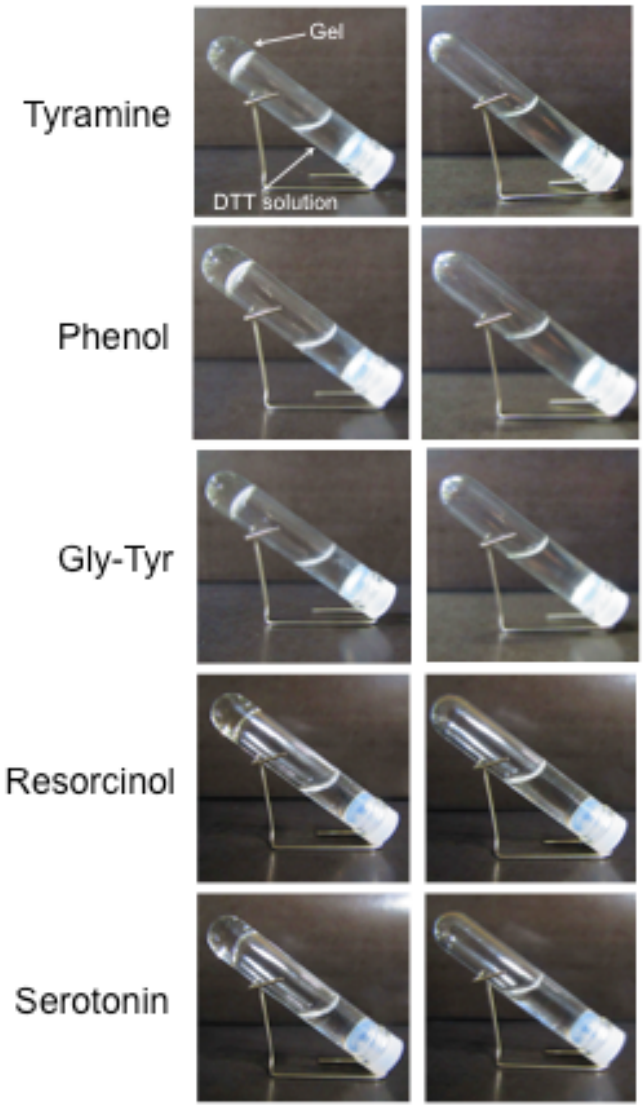

Fig. S4. Photographs of 4-arm PEG-SH hydrogels (left) just after and (right) 60 min after soaking in DTT solution.

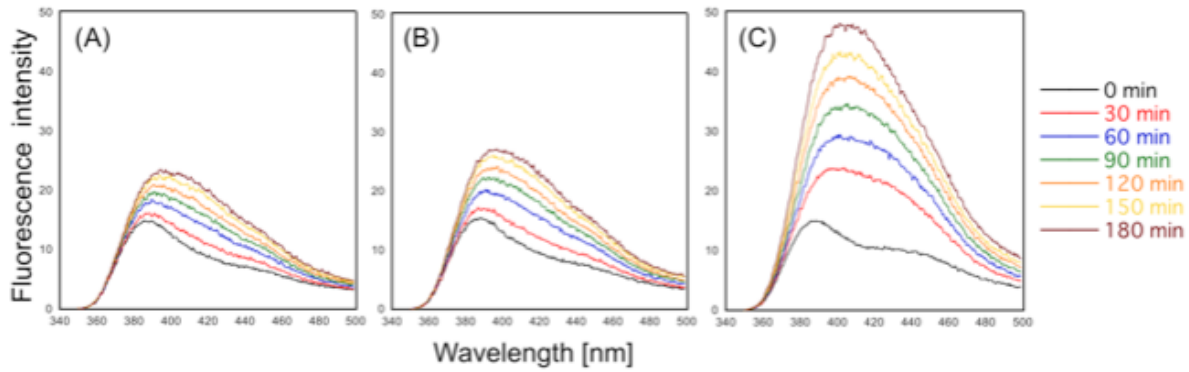

Fig. S5. Effect of HRP concentrations of (A) 5, (B) 10, and (C) 50 units/mL, on fluorescence, after mixing the chemicals, GSH, tyramine hydrochloride and HRP.

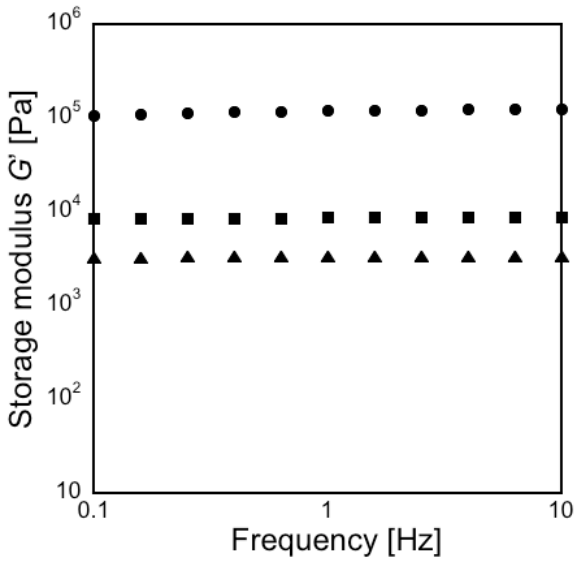

Fig. S6. Storage modulus (*G*') of 4-arm PEG-SH hydrogel, 4-arm PEG-SH concentration: (●) 15, (■) 10 and (▲) 5 wt%.

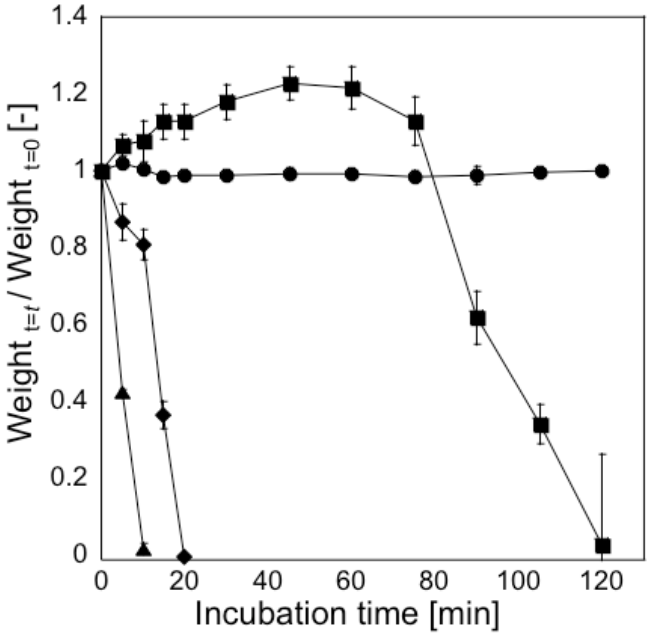

Fig. S7. Weight change profiles of PEG-SH hydrogels soaked in PBS solution containing Cys at (●) 0, (■) 1, (♦) 5 and (▲) 10 mM. Bars indicate standard deviations (n = 3).