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A redox-responsive hydrogel composed of poly(ethylene glycol) and gelatin was prepared via an enzymatic oxidative reaction. Fibroblast cells adhered on the hydrogel showed proliferation and they could be recovered as a cell sheet by degradation of the scaffold under mild reductive conditions, thus providing a novel platform for cell culture.

Hydrogels have been studied intensively as tissue engineering scaffolds because of their excellent properties, such as high water content, good biocompatibility and high permeability to nutrients and metabolites.[1, 2] In recent years, a variety of hydrogels comprising both synthetic and naturally derived polymers have been studied to create precise microenvironmental conditions for cellular growth. Cell fate and function can be influenced by physicochemical properties of the hydrogel, such as swelling ratio and mechanical properties.[3–5] Therefore, the ability to manipulate the physicochemical properties of hydrogel is favorable to the development of tissue engineering scaffolds. Synthetic polymer-based hydrogels such as poly(ethylene glycol) (PEG), poly(lactic acid), poly(glycolic acid) and poly(vinyl alcohol) have been investigated for use as tissue engineering scaffolds. One advantage of using synthetic polymers is the ease of adjusting the physicochemical properties of the hydrogel including swelling ratio and mechanical strength.[6] In particular, PEG has been one of the most commonly used materials for biomedical applications because of its nontoxicity, good biocompatibility and non-immunogenicity; however, as PEG exhibits minimal or no intrinsic biological activity because of its non-adhesive nature, the development of a PEG-based hydrogel as a tissue engineering scaffold has been limited. However, PEG-based hydrogels functionalized with natural polymers such as collagen, gelatin and hyaluronic acid have recently been reported with the purpose of regenerating various tissues including cartilage, bone, nerve, vasculature and muscle.[7–10]

We have recently reported a new enzymatic method for the preparation of a redox-sensitive PEG hydrogel using horseradish peroxidase (HRP) as a biocatalyst.[11] HRP is an enzyme that efficiently catalyzes the oxidative radical coupling of phenol, aniline and thiol derivatives using hydrogen peroxide (H_2O_2). In general, aqueous H_2O_2 solution is directly added to a reaction system to activate HRP, followed by hydrogelation of the polymer solution.[12–14] However, the immediate high local concentration of H_2O_2 upon addition may negatively affect the activity of biological entities encapsulated in the hydrogel. We showed that the PEG hydrogel possessing redox-responsiveness could be prepared by only mixing thiolated PEG (PEG-SH), HRP and phenolic compounds without exogenous H_2O_2.[11] This gelation system is mild enough to retain the activity of biological entities because H_2O_2 molecules, which are needed to activate HRP, are generated gradually and rapidly consumed by HRP in solution. The resulting hydrogel encapsulated cells and retained their viability.

Here we attempted the functionalization of a PEG hydrogel using gelatin as a bioactive component. Gelatin is a naturally occurring polymer produced by the partial hydrolysis of collagen and gelatin-based materials are commonly used for biopharmaceutical and biomedical applications because of their excellent biocompatibility. Cell-binding motifs such as the RGD sequence, which is essential for cellular proliferation, are retained by gelatin. To incorporate this polymer into the PEG hydrogel, we synthesized thiol-modified gelatin (Gela-SH) using a carbodiimide-mediated amide formation between the carboxyl groups of gelatin

and the amine groups of cystamine. The degree of substitution (DS) of Gela-SH determined by Ellman's test[15] was c.a. 0.45 mmol−SH/g−gelatin.

Table 1. Sample composition in the preparation of (PEG-SH)-(Gela-SH) hydrogels.

Samples	PEG-SH [% (w/v)]	Gela-SH [% (w/v)]
P5G0	5	0
P5G0.01	5	0.01
P5G0.1	5	0.1
P2.5G0.1	2.5	0.1
P10G0.1	10	0.1

HRP-catalyzed hydrogelation using PEG-SH and Gela-SH was conducted and the physicochemical properties of the resultant (PEG-SH)-(Gela-SH) hydrogels were evaluated by varying the concentrations of PEG-SH (2.5–10% (w/v)) and Gela-SH (0–0.1% (w/v)) (Table 1). The swelling ratio of hydrogels significantly changed within 24 h of incubation in PBS and then reached a plateau for all hydrogels (Figure S2). Equilibrium swelling ratio (Q_M) decreased and storage modulus (G') increased with increasing PEG-SH concentration (Table 2, Figure 1). For example, when the PEG-SH concentration was increased from 2.5% (w/v) to 10% (w/v) at the Gela-SH concentration of 0.1% (w/v), the Q_M value decreased by almost half and the G' value increased 10-fold. In general, the Q_M and G' values of a hydrogel are associated with its cross-link density; Q_M decreases and G' increases by increasing the cross-link density of hydrogel. Under the conditions in this study, gel content was >80% for all hydrogels prepared (Table 2), suggesting that the cross-link reaction proceeded sufficiently under each of the hydrogelation conditions. Since the thiol concentration of gel precursor solutions increases as the PEG-SH concentration increases, the cross-link density of the (PEG-SH)-(Gela-SH) hydrogel also increases with increasing PEG-SH concentration, which is attributed to the change in the Q_M value and G' of (PEG-SH)-(Gela-SH) hydrogel.

On the other hand, Q_M and G' of hydrogel prepared from 5% (w/v) PEG-SH showed little variation with changing Gela-SH concentrations (c.a. 40 and 2500 Pa, respectively) (Table 2 and Figure 1). It is worth noting that the concentrations of Gela-SH were significantly lower than that of PEG-SH in this experiment. The total thiol concentrations of gel precursor solutions were not changed significantly in each condition (Table S1). In contrast to the previous reports, where the physicochemical properties of hydrogels composed of PEG and gelatin changed by varying the concentration of both polymers,[13, 16] the physicochemical properties of the obtained hydrogel depended mostly on the concentration of PEG-SH. These results demonstrate that the physicochemical properties of (PEG-SH)-(Gela-SH) hydrogel can be easily controlled by changing the PEG-SH concentration when the Gela-SH is set at a relatively low concentration.

Table 2. Equilibrium swelling ratio (Q_M) and gel content of (PEG-SH)-(Gela-SH) hydrogels. Data are presented as mean ± standard deviation (n = 3).

Samples	Q_M [a.u.]	Gel content [%]
P5G0	41.1 ± 1.4	81.5 ± 2.6
P5G0.01	39.6 ± 0.3	84.6 ± 1.0
P5G0.1	39.1 ± 1.0	85.0 ± 2.6
P2.5G0.1	64.2 ± 5.9	82.1 ± 4.9
P10G0.1	33.6 ± 0.6	88.9 ± 1.2

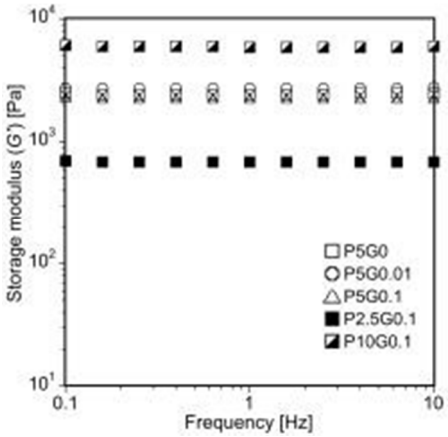

Figure 1. Storage modulus (G') of (PEG-SH)-(Gela-SH) hydrogel under various conditions.

The ability of cells to attach, spread and proliferate on hydrogel is an important attribute for tissue engineering. We next evaluated the cellular adhesiveness and proliferation of L929 fibroblast cells on (PEG-SH)-(Gela-SH) hydrogels. Figure S3 shows L929 cells on the hydrogel after 4 h of cell seeding. Adhered and spread cells were observed on the (PEG-SH)-(Gela-SH) hydrogels (Figure S3 (B–E)), while adherent cells were rarely observed on the PEG-SH hydrogel without Gela-SH (P5G0) (Figure S3 (A)). In addition, almost no adherent cells were observed on the hydrogel prepared using PEG-SH and native gelatin (without thiol moieties) (Figure S4). This result was attributed to the fact that the natural gelatin has no intrinsic cysteine that can cross-link with PEG-SH. These results clearly demonstrated that the PEG-SH hydrogel was imparted with the cellular adhesiveness by co-cross-linking with thiol-containing gelatin and also suggests that a range of bioactive entities such as polysaccharide, peptide, enzyme and growth factor could be immobilized in PEG-SH hydrogel in the same manner. The number of adhered fibroblast cells on the (PEG-SH)-(Gela-SH) hydrogel increased with increasing Gela-SH concentration at a given PEG-SH concentration (5% (w/v)) (Figure 2(A)). In addition, >95% of seeded cells were adhered on the P5G0.1 hydrogel after 4 h of incubation, which showed the same level as the gelatin-coated dish used as a positive control. Furthermore, a number of cells on P5G0.1 hydrogel after 5 d of cultivation increased by c.a. 40% compared with that of P5G0.01 hydrogel (Figure 2(B)). This result indicated that the rate of cell growth on P5G0.1 hydrogel was slightly faster than that of P5G0.01 hydrogel, i.e. cell growth increased with increasing gelatin-SH. The faster rate of cell growth was also confirmed by cell density on the (PEG-SH)-(Gela-SH) hydrogels at 5 d of cultivation; cell density on P5G0.1 hydrogel was slightly higher than that of P5G0.01 hydrogel (Figure S5(B, D)). In addition, growth profile and morphology of L929 cells on P5G0.1 hydrogel

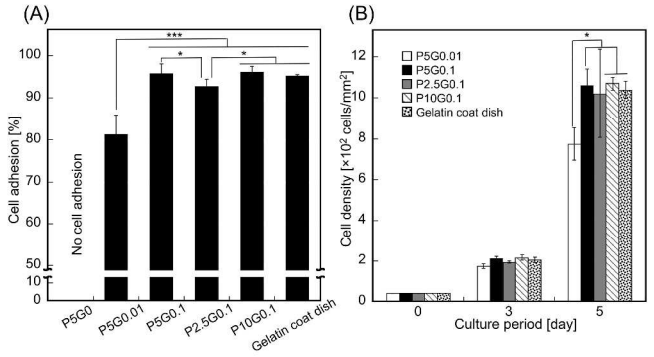

Figure 2. (A) Cellular adhesiveness and (B) growth profiles of L929 cells on (PEG-SH)-(Gela-SH) hydrogels. Error bars denote standard deviations (SD) (n = 6 (A) and 3 (B)) (*p < 0.05 and ***p < 0.001).

showed the same trend as that of the gelatin-coated dish (Figure 2(B) and Figure S5).

In terms of PEG-SH concentration, the cellular adhesiveness was more than 90% for all hydrogels prepared in the presence of 0.1% (w/v) Gela-SH. However, P2.5G0.1 hydrogel exhibited slightly lower adhesiveness than other hydrogels (Figure 2(A), *p<0.05), though the cells showed similar morphology (Figure S3(C–F)). It has been reported that the cellular adhesiveness is affected by the mechanical properties of a hydrogel e.g., stiffer hydrogels show greater cellular attachment.[16, 17] We found that the storage modulus of P2.5G0.1 hydrogel was c.a. 10 times lower than that of P10G0.1 hydrogel. This result may be attributed to the decrease in the mechanical properties of (PEG-SH)-(Gela-SH) hydrogels. However, there was no significant difference in the growth profile and morphology of L929 cells on (PEG-SH)-(Gela-SH) hydrogels that showed dependence on PEG-SH concentration. Thus, it is possible that the slight difference in the adhesiveness at the early stage has little effect on the proliferation of fibroblast cells. Based on these results, the following experiments were carried out using P5G0.1 hydrogel because of the excellent cellular adhesiveness and proliferation.

Redox-responsive hydrogels are attractive as a cell culture platform for tissue engineering because the template scaffold can be degraded at the desired time under mild reductive conditions.[18] We have demonstrated that PEG-SH hydrogel prepared by HRP catalysis can be degraded using cysteine (Cys) solution under conditions non-toxic to mammalian cells.[11] We therefore evaluated the potential of the obtained (PEG-SH)-(Gela-SH) hydrogels for a cell sheet technology. The cell sheet technology is an innovative approach to fabricate functional tissue-like structures using 2D interconnected cells.[19, 20] In this study, we used (PEG-SH)-(Gela-SH) hydrogels as a cell culture platform for preparation of a cell sheet. L929 cells were cultivated on P5G0.1 hydrogel until the confluent monolayer was attained. Subsequently, 10 mM Cys solution was poured onto the hydrogel. After 30 min of incubation, complete detachment of the cell sheet was observed (Figure 3(A, B)). The cell sheet was re-seeded on a fresh cell culture dish and cultivated under standard conditions (37°C, 5% CO_2) (Figure 3(C–F) and Figure S6). After 24 h of cultivation, living and dead cells were stained with green and red fluorescence, respectively, using a

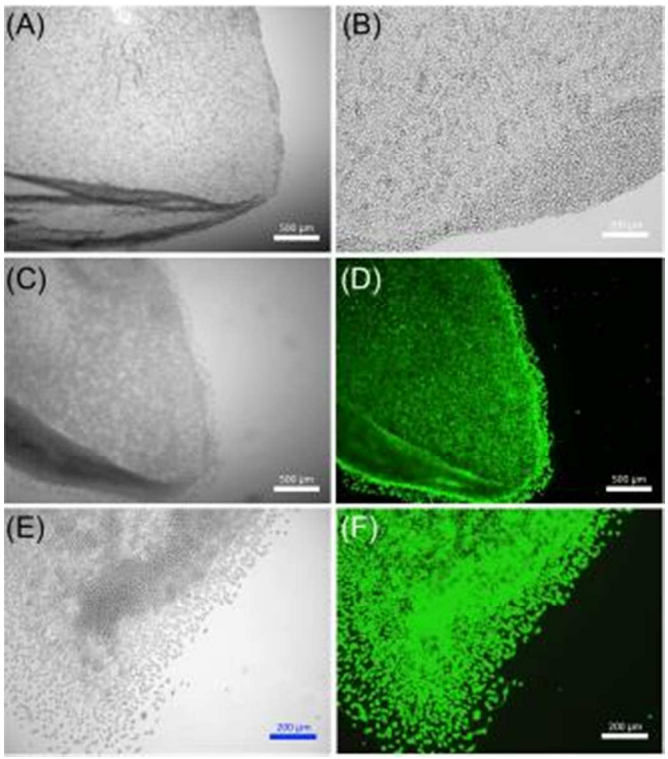

Figure 3. Photomicrographs of L929 cell sheet (A and B) detached from (PEG-SH)-(Gela-SH) hydrogel 30 min after addition of Cys solution, and (C–F) 24 h after transferring to a fresh cell culture dish. (A–C and E) Brightfield images and (D, F) fluorescence images, respectively. Bars: (A, C and D) 500 μm; (B, E and F) 200 μm.

fluorescence double-staining kit. Figure 3(D, F) and Figure S5(B) show fluorescence images of the cell sheet on the cell culture dish. Dead cells (red fluorescence) were not clearly observed after 24 h of incubation (Figure 3(D, F) and Figure S6(B)), indicating that most of the L929 cells in the cell sheet were alive. In addition, L929 cells at the edge of the cell sheet adhered and spread on the cell culture dish (Figure 3(E, F)). Importantly, the Cys solution degraded only the (PEG-SH)-(Gela-SH) hydrogel without lowering cell viability or breaking cell-to-cell junctions. An advantage of using (PEG-SH)-(Gela-SH) hydrogel is that the cell sheet can be harvested at the desired time simply by adding Cys solution. These results clearly demonstrated the feasibility of (PEG-SH)-(Gela-SH) hydrogel as substrates for adherent cell culture.

Conclusion

In conclusion, we succeeded in the preparation of a hydrogel composed of PEG and gelatin, using a HRP-catalyzed reaction without the need for exogenous H_2O_2. Physicochemical properties of obtained (PEG-SH)-(Gela-SH) hydrogels could be easily controlled by changing PEG-SH concentrations under the conditions used in this study. Cellular adhesiveness and proliferation rate on the PEG-SH hydrogel increased by conjugation with Gela-SH, and P5G0.1 hydrogel showed the same level of proliferation as that of the gelatin-coated dish. Furthermore, confluent cells on the (PEG-SH)-(Gela-SH) hydrogel could be harvested within 30 min using a non-toxic detachment process by degrading the redox-sensitive hydrogel with Cys solution. We believe that the (PEG-SH)-(Gela-

SH) hydrogel has great potential for biomedical and bioengineering applications, especially as a tissue engineering scaffold.

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Notes and references

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† Electronic Supplementary Information (ESI) available.

1. O. D. Krishna, K. L. Kiick, *Biopolymers*, 2010, 94, 32
2. A. S. Hoffman, *Adv. Drug Deliv. Rev.*, 2002, 54, 3
3. R. W. Sands, D. J. Mooney, *Curr. Opin. Biotechnol.*, 2007, 18, 448
4. D. Steliktar, *Science*, 2012, 336, 1124
5. J. L. Drury, D. J. Mooney, *Biomaterials*, 2003, 24, 4337
6. J. Zhu, *Biomaterials*, 2010, 31, 4639
7. A, Skardal, J. Zhang, G. D. Prestwich, *Biomaterials*, 2010, 31, 6173
8. S. R. Peyton, C. B. Raub, V. P. Keschrumrus, A. J. Putnam, *Biomaterials*, 2006, 27, 4881
9. T. Taguchi, L. Xu, H. Kobayashi, A. Taniguchi, K. Kataoka, J. Tanaka, *Biomaterials*, 2005, 26, 1247
10. R. Scott, L. Marqurdt, R. K. Willits, J. Biomed. Mater. A, 2010, 93, 817
11. K. Moriyama, K. Minamihata, R. Wakabayashi, M. Goto, H. Kamiya, *Chem. Commun.*, 2014, 50, 5895
12. K. M. Park, Y. M. Shin, Y. K. Joung, H. Shin, K. D. Park, *Biomacromolecules*, 2010, 11, 706
13. K. M. Park, Y. Lee, J. Y. Son, D. H. Oh, J. S. Lee, K. D. Park, *Biomacromolecules*, 2012, 13, 604
14. D. J. Menzies, A. Cameron, T. Munro, E. Wolvetang, L. Grondahl, J. J. Cooper–White, *Biomacromolecules*, 2013, 14, 413
15. G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, 82, 70
16. C. B. Hutson, J. W. Nichol, H. Aubin, H. Bae, S. Yamanlar, S. Al-Haque, S. T. Koshy, S. Khademhosseini, Tssue Eng. Part A, 2011, 17, 1713
17. S. Ouasti, R. Donno, F. Cellesi, M. J. Sherratt, G. Terenghi, N. Tirelli, *Biomaterials*, 2011, 32, 6456
18. M. Matsusaki, H. Yoshida, M, Akashi, *Biomaterials*, 2007, 28, 2729
19. J. Yang, M. Yamato, T. Shimizu, H. Sekine, K. Ohashi, M. Kanzaki, T. Ohki, K. Nishida, T. Okano, *Biomaterials*, 2007, 28, 5033
20. I. E. Hannachi, M. Yamato, T. Okano, *Biofabrication*, 2009, 1, 022002

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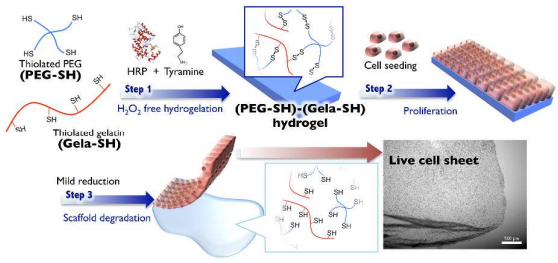

Horseradish peroxidase-mediated preparation of redox-responsive hydrogels composed of thiolated poly(ethylene glycol) and gelatin allowed the proliferation of cells on the gel and the harvest as a viable cell sheet.