

Activated-Ester-Type Photocleavable Crosslinker for Preparation of Photodegradable Hydrogels Using a Two-Component Mixing Reaction

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Photodegradable hydrogels have emerged as powerful platforms for studying and directing cellular behavior in a spatiotemporally controlled manner. Photodegradable hydrogels have previously been formed by free radical polymerizations, Michael-type addition reactions, and orthogonal click reactions. Here, an ester-activated photocleavable crosslinker is presented for preparing photodegradable hydrogels by means of a one-step mixing reaction between the crosslinker and a biocompatible polymer containing amino moieties (amino-terminated tetra-arm poly(ethylene glycol) or gelatin). It is demonstrated that photodegradable hydrogels micropatterned by photolithography can be used to culture cells with high viability and proliferation rates. The resulting micropatterned cell-laden structures can potentially be used to create 3D biomaterials for various tissue-engineering applications.

1. Introduction

Micropatterned hydrogels have been used to engineer 3D tissue constructs as in vitro models that closely mimic human organs and tissues. To fabricate particular microstructures, several technologies have been developed, including top-down and bottom-up approaches.^[1–5] For example, photocrosslinked polymers are widely used to control not only the microstructure and stiffness of the hydrogel but also the cellular behavior on and within it. Various research groups have

generated micropatterned hydrogels for tissue-engineering applications with photo-crosslinked polymers, including derivatives of poly(ethylene glycol) (PEG),^[6–8] polysaccharides,^[9–11] and proteins (e.g., gelatin).^[12,13]

Recently, photodegradable hydrogels have attracted significant attention because of their tunable mechanical and chemical properties and because they are suitable for the creation of 3D microstructures^[14,15] for biomaterials and tissue-engineering research.^[16] Such photodegradable hydrogels have become highly popular in these fields.^[17,18] The physical and chemical properties of photodegradable hydrogels can be both temporally and spatially

controlled by light irradiation (single and two photons),^[19,20] and this process is also compatible with living cells.^[14,21]

To date, despite the many reported approaches, current methods to prepare photodegradable hydrogels are restricted to reactions between synthetic molecules, such as radical reactions,^[14,20,22–25] Michael-type conjugations,^[26] and orthogonal click reactions.^[15,27] Among these reactions, multicomponent mixing reactions enable the reaction of two or more molecular-scale components, where the gelation takes place spontaneously after mixing. Such systems are highly tunable, as

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either component can be easily modified to alter the hydrogel performance or to introduce additional functionalities. In previous studies, Michael-type conjugations^[27] and orthogonal click reactions^[15,26] were used to fabricate photodegradable hydrogels in multicomponent mixing reactions. These reactions, however, require chemical modification of the component materials. In addition, most of the resulting photodegradable hydrogels have been derivatives of PEG or polysaccharides,^[15,27] which are difficult for cells to degrade or have low adhesion. The lack of cell-responsive features may affect the viability and metabolic activity of cells and greatly limits the culture conditions. Although cell-binding sequences such as Arg-Gly-Asp and other peptides can be incorporated into photodegradable hydrogels by using a combination of synthetic homo- and heterofunctional crosslinkers,^[14,15] development of simple systems to prepare biologically functional photodegradable hydrogels would facilitate more widespread use of this technology. Here, we present an activated-ester-type photocleavable crosslinker that facilitates the formation of photodegradable hydrogels by means of a one-step, two-component mixing reaction with a biocompatible polymer containing amino moieties (amino-terminated tetra-arm PEG (amino-4-arm PEG) or gelatin). The resulting photodegradable hydrogels were micropatterned to form microscale structures. In addition, we demonstrated that cells can be cultured on photodegradable hydrogels prepared with either amino-4-arm PEG or gelatin.

2. Results and Discussion

2.1. Characteristics of Photocleavable Crosslinker and Photodegradable Hydrogels

We first synthesized our *N*-hydroxysuccinimide-terminated photocleavable crosslinker (NHS-PC-4-arm PEG, $M_w = 12\,062$) crosslinker, which is composed of the following functional groups (Figure 1): i) PEG as a water-soluble main polymer chain; ii) *o*-nitrobenzyl groups, which are cleaved by light irradiation; and iii) NHS-activated ester groups, which react with primary amine groups to form peptide bonds under physiological conditions (e.g., pH 7.4, 37 °C). The cleavage behavior of the crosslinker in an aqueous solution was evaluated by means of absorption spectroscopy (Figure 2a). Light exposure energy at 0.3 J cm^{-2} induced cleavage of the *o*-nitrobenzyl groups, as evidenced by an increase in the absorbance at 390 nm. To estimate the molar ratio of the uncleaved crosslinker to its cleaved analog after light irradiation, the exposed sample was also investigated by performing ^1H NMR spectroscopy. Figure S4 (Supporting Information) shows an image of aqueous solution of NHS-PC-4-arm PEG after light exposure in NMR tubes. The amount of uncleaved crosslinker gradually decreased as a function of the light exposure energy (Figure 2b; NMR spectra are available in Figure S5, Supporting Information).

To prepare photodegradable hydrogels from amino-4-arm PEG or gelatin, a $10 \times 10^{-3}\text{ M}$ solution of NHS-PC-4-arm PEG

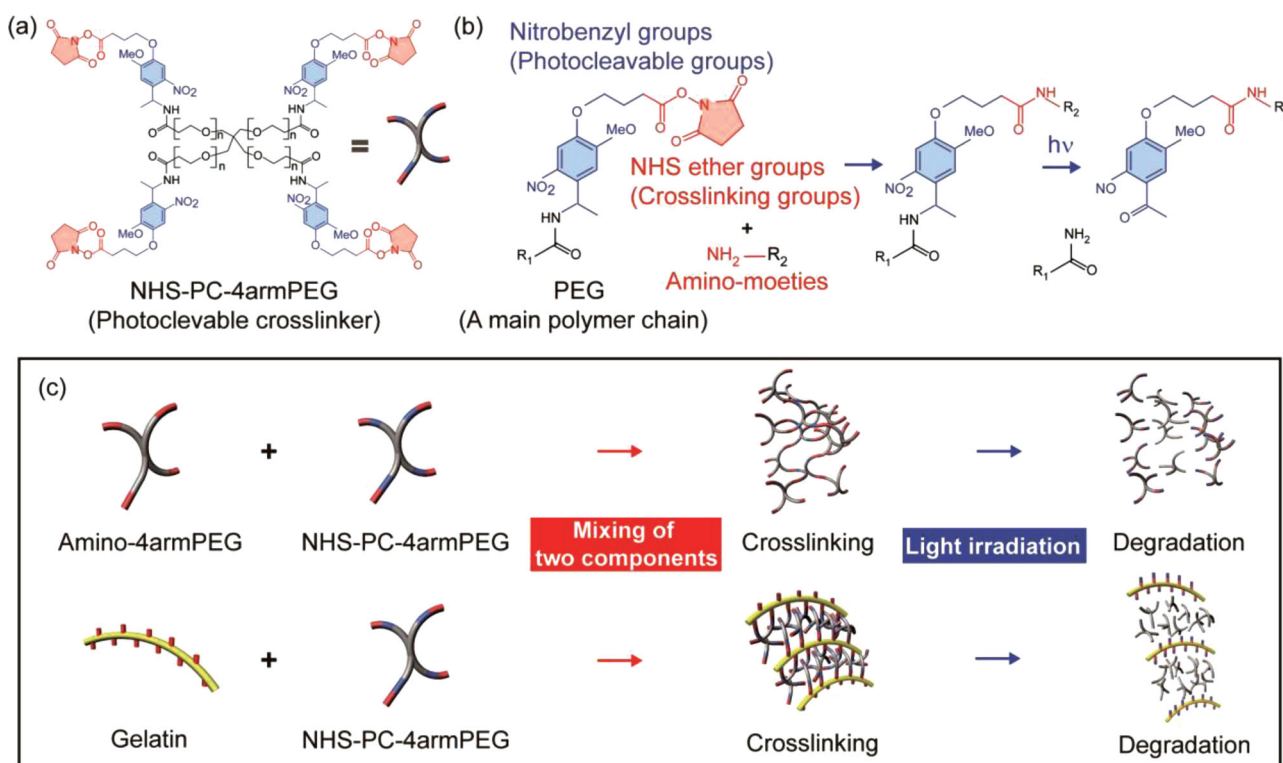


Figure 1. Schematic diagram of hydrogel formation by crosslinking of amine-containing polymers and subsequent photodegradation by light irradiation. a) Scheme for NHS-PC-4-arm PEG. b) Crosslinking of NHS-PC-4-arm PEG with primary amines and subsequent photocleavage. c) Schematic representation of the overall process for formation and degradation of photodegradable hydrogels prepared with amino-4-arm PEG and gelatin.

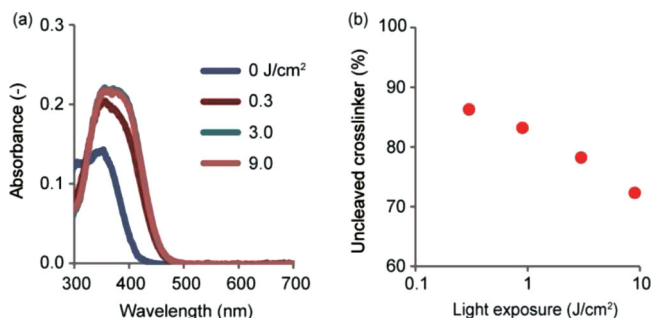


Figure 2. Influence of light irradiation (365 nm , 30 mW cm^{-2}) on the absorption spectrum and cleavage behavior of the photocleavable crosslinker. a) Absorption spectra of 0.01% (w/v) aqueous solution of NHS-PC-4-arm PEG before and after irradiation in a quartz cell (1 cm path length). b) Dependence of the molar ratio of uncleaved to cleaved NHS-PC-4-arm PEG (1.25% w/v) on light exposure energy after irradiation in deuterated water in an NMR tube (4 mm diameter).

crosslinker in $10 \times 10^{-3}\text{ M}$ phthalic acid buffer (pH 4.0) with $140 \times 10^{-3}\text{ M}$ NaCl was allowed to react with a prepolymer solution containing $10 \times 10^{-3}\text{ M}$ amino-4-arm PEG or 5% (w/v) gelatin in a 1:1 (v/v) mixture of PBS and 0.3 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.0). The appropriate concentration ranges of the main polymer and synthesized NHS-PC-4-arm PEG for hydrogel formation were also investigated (hydrogel formation results are available in Table S1 and S2, Supporting Information). According to our experimental results, the concentration range of NHS-PC-4-arm PEG suitable for hydrogel formation with gelatin as a main polymer was wider than that obtained for amino-4-arm

PEG, suggesting that the long main polymer chain of gelatin ($M_w = 50\,000\text{--}100\,000$ according to the manufacturer's information) could allow hydrogels to form at low concentrations of NHS-PC-4-arm PEG.

Generally, an NHS-activated ester group is highly reactive with primary amine groups to form peptide bonds under neutral to higher pH conditions, and the reaction rate can be controlled by changing the pH of the solution.^[28] To investigate the gelation times for different pH values, hydrogels were prepared using HEPES buffers with different pH values (pH range: from 6.6 to 7.0). The time profiles of the elastic modulus (G') and viscous modulus (G'') were measured using a rheometer, and the gelation time was estimated as their crossover point (CP) (Figure 3). For the samples prepared using the pH 7.0 HEPES buffer, the G' and G'' values for both amino-4-arm PEG and gelatin were measured, and the G'' values were found to be higher than the G' values (Figure 3a,d). Thus, the crosslinking reaction rate was rapid, leading to gelation within 30 s. For the pH 6.6 and 6.8 HEPES buffers, the samples were still in a liquid state at the beginning of crosslinking, G' was larger than G'' , and viscous properties dominated (Figures 3b,c,e, and f). As the solutions began to become gel-like because of the formation of the crosslinked network, both G' and G'' increased in a sigmoidal manner. In particular, in the case of the gelatin prepolymer solutions prepared using pH 6.6 and 6.8 HEPES buffers, the crosslinking reaction rates were slow, and homogeneous hydrogel formation required more than a few hours. These results suggest that the NHS moiety is stable under acidic conditions but reacts quickly with primary amines at neutral pH, as expected. On the other hand, significant increases in gelation time were observed for the gelatin solutions prepared using pH

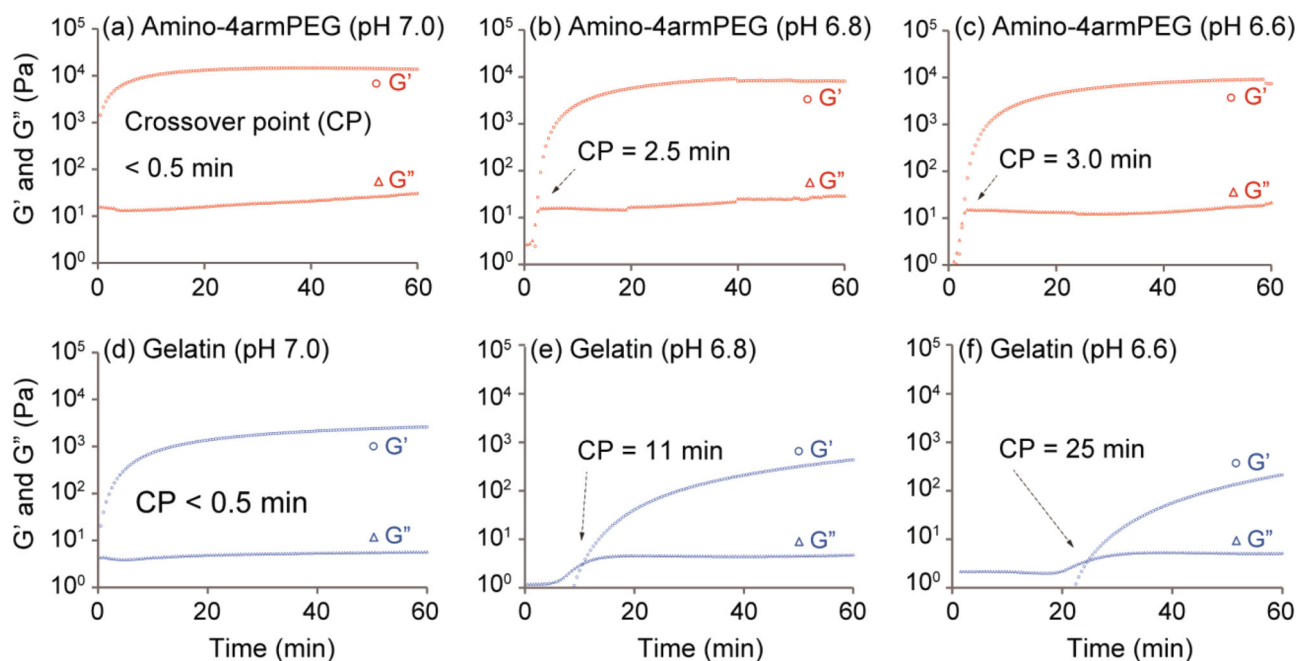


Figure 3. Time sweep profiles of elastic modulus (G') and viscous modulus (G'') around the gelation point at $37\text{ }^\circ\text{C}$. Gelation time was determined as the time at which G' and G'' intersect (crossover point, CP). a–c) Gelation profile of $5 \times 10^{-3}\text{ M}$ amino-4-arm PEG and $5 \times 10^{-3}\text{ M}$ NHS-PC-4-arm PEG (5PEG+5CL). d–f) Gelation profile obtained by using 2.5% (w/v) gelatin and 5.0% (w/v) NHS-PC-4-arm PEG (2.5GL+5CL). The pH values in the figure are those of the HEPES buffers used for preparation of the prepolymer solution.

6.6 and 6.8 HEPES buffers (Figures 3e,f). Because gelatin is a polyampholyte, the pH of the prepolymer solution was different from that of the HEPES buffer, and the final pH of the mixed solution dominated the reaction rate, which delayed the crosslinking reaction. (Changes in the pH values of the solution before and after the two components were mixed are listed in Table S3 and S4, Supporting Information.) An additional note on hydrogel preparation is that the viscosity of the solution and inter- or intramolecular interactions, especially in the case of gelatin, may affect the gelation time.

2.2. Evaluation of Morphology and Degradation Behavior of Micropatterned Photodegradable Hydrogels

Photodegradable hydrogels were synthesized using the photocleavable crosslinker in a one-step mixing reaction for both amino-4-arm PEG and gelatin. To create the micropatterns,

the formed hydrogels were irradiated with light (365 nm, 30 mW cm^{-2}) through photomasks. After irradiation, the color in the irradiated region changed slightly, as was observed for the crosslinker solution (Figure S4, Supporting Information). To erode the degraded polymer in the exposed regions of the hydrogels, the irradiated samples were immersed in PBS for a day at 37°C (Figure S3, Supporting Information). Micropatterns corresponding to the photomasks were created by the erosion process. Both lines and circles could be precisely created, and the resolution varied from 20 to $500 \mu\text{m}$ (Figure 4a–d). Unexposed regions remained intact. Although the concentration range for micropatterning of hydrogels prepared with gelatin as the main polymer was wider than that for micropatterning of hydrogels prepared with amino-4-arm PEG (hydrogel formation results are listed in Table S1 and S2, Supporting Information), the resolution of the micropatterning did not substantially differ between the hydrogels prepared with amino-4-arm PEG (Figure 4a,c) or with gelatin (Figure 4b,d). In

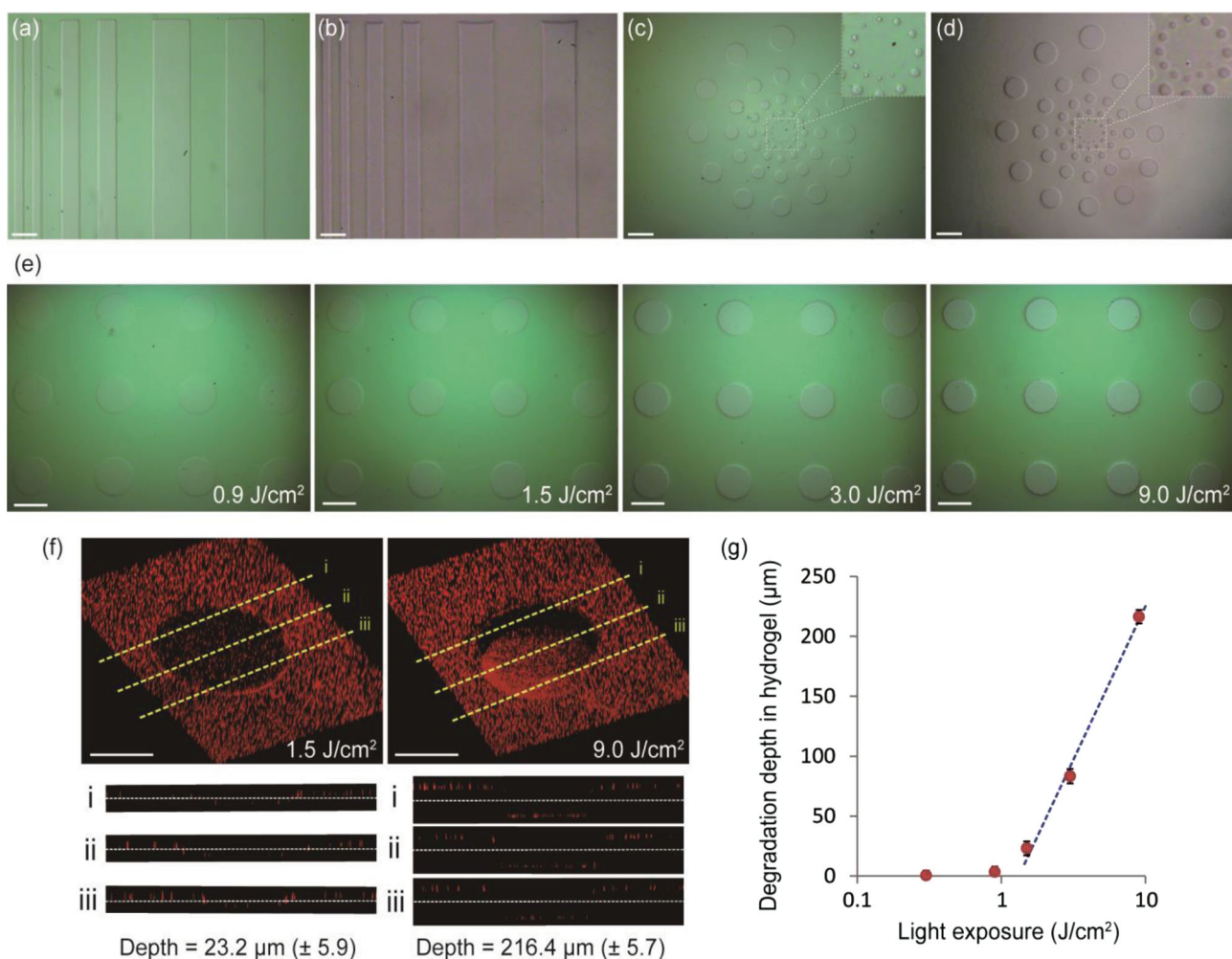


Figure 4. Micropatterning of photodegradable hydrogels by light irradiation through photomasks. a–d) Microscopic images of micropatterned photodegradable hydrogels composed of a,c) $5 \times 10^{-3} \text{ M}$ amino-4-arm PEG with $5 \times 10^{-3} \text{ M}$ NHS-PC-4-arm PEG (5PEG+5CL) and b,d) 2.5% (w/v) gelatin with 5.0% (w/v) NHS-PC-4-arm PEG (2.5GL+5CL). Irradiation of the hydrogel through the photomask induced degradation of the exposed regions and generated a,b) lines or c,d) circles. e) 2D micrographs and f) 3D confocal micrographs of micropatterned hydrogels prepared with amino-4-arm PEG. a,c,e) For visualization, patterned hydrogels prepared with amino-4-arm PEG were stained with CBB solution for 1 h before the images were captured. Scale bars = $500 \mu\text{m}$. g) Effect of light exposure energy on degradation depth. Error bars indicate standard deviations for four samples.

addition, resolution of micropatterning depends on the thickness of hydrogels. Generally, creation of small structure in thick hydrogels with aspect ratios higher than five is difficult because of depth of focus, that is, distance from the photomask, and swelling of hydrogels. The effects of swelling were more significant for the smaller photomask pattern on the thicker hydrogel. In addition, the effects of swelling were different in different geometry on photomask patterns (lines or circles).

An additional note on the micropatterning of hydrogels is that the time required for erosion depends on the thickness of the hydrogel, concentrations of the crosslinker, and amine-containing polymer, and light exposure conditions. In general, thick hydrogels prepared with high concentrations of the crosslinker and polymer require longer erosion times. The effect of the light exposure energy on the degradation depth in the micropatterned hydrogels prepared with amino-4-arm PEG was also evaluated. The visibility of the micropatterns corresponding to the photomask increased with increasing light exposure energy (Figure 4e). To evaluate the degradation depth, the surface profile and morphology of the micropatterned hydrogels were characterized using a confocal laser scanning microscope (Figure 4f), and the degradation depth in the x - z plane was measured (Figure 4g). Although the absorbance and NMR spectra indicated that the crosslinker was cleaved at light exposure energies of $<1.2 \text{ J cm}^{-2}$, significant degradation of the hydrogel was not observed. Under these conditions, photocleavage of the crosslinker induced a decrease in crosslinking density but was insufficient for complete hydrogel degradation. After irradiation at $>1.2 \text{ J cm}^{-2}$, hydrogel degradation was clearly observed, and the degradation depth increased linearly with the log of the exposure energy. This linear relationship can be explained by the Beer-Lambert law,^[29] in which the log of the transmitted intensity is proportional to the depth from the sample surface. The transmitted intensity is defined as

$$\log_{10}\left(\frac{I}{I_0}\right) = \epsilon cl \quad (1)$$

where I and I_0 are the light intensity at the given depth (l) and sample surface, respectively, ϵ is the molar adsorption coefficient, and c is the concentration of the solution. On the basis of this correlation, the degradation depth of the photodegradable hydrogels can be easily controlled by varying the light exposure, as can the shape and size of the degraded regions.

2.3. Characterization of Cell Behavior on Photodegradable Hydrogels

Finally, as a proof of concept for cell manipulation on the photodegradable hydrogels prepared using our photocleavable crosslinker, human umbilical vein endothelial cells (HUVECs) were seeded as model cells onto the hydrogels prepared with amino-4-arm PEG or gelatin as the main polymer. As expected, few cells adhered onto hydrogel surfaces prepared with amino-4-arm PEG, because PEG is inert to cell adhesion^[13] (Figure S6, Supporting Information). HUVECs readily adhered only to the hydrogel surface prepared with gelatin (Figure 5a,b). Cells on both the "2.5GL+1CL" and "2.5GL+3CL" samples elongated and

proliferated over 3 d, and significant differences in cell growth were observed on the hydrogels with 1.0%–5.0% (w/v) photocleavable crosslinker. Hence, the differences in cell behavior (i.e., cell adhesion, cell elongation, and cell proliferation) on the hydrogels prepared with gelatin stemmed from the concentration of the photocleavable crosslinker. In addition, a similar phenomenon was observed for the hydrogel prepared with 1.25% (w/v) gelatin: higher cell growth was observed on the hydrogel with 0.5% (w/v) photocleavable crosslinker than on that with 2.5% (w/v) photocleavable crosslinker. (For the results for hydrogels prepared with 1.25% (w/v) gelatin, see Figure S7a,b, Supporting Information). These results suggest that the free amino acid residues in the gelatin related to cell binding are probably important for cell adhesion and proliferation, because free amino acid residues remain intact at lower crosslinker concentrations.

To demonstrate the cell patterning, HUVECs on the photodegradable hydrogels prepared with gelatin (2.5GL+1CL) were irradiated with a PC-controlled microprojection system after 3 d of culture. For light exposures energies of $>2.0 \text{ J cm}^{-2}$, the cells fell into the pores generated in the hydrogels and moved from the center to the periphery owing to hydrogel erosion after the micropattern was developed (Figure 6c,d). The resulting cell patterns reflected hydrogel degradation. Hence, cell patterns were realized on photodegradable hydrogels with microscale precision using a procedure similar to the hydrogel patterning described above. To evaluate the effects of light irradiation and hydrogel degradation on the cell viability, a live/dead assay was performed as well. After light irradiation of the elongated cells, the cells were stained by Calcein and EthD-1. The cell viability in the exposed regions was similar to that in the unexposed regions (Figures 6b–d), demonstrating that light irradiation and hydrogel degradation did not alter cell viability during this process. In addition, the cell growth was supported on the bottom surface in the exposed regions within 1–2 d after light irradiation (Figure S8, Supporting Information).

In this study, we have demonstrated a one-step, two-component mixing approach to preparing photodegradable hydrogels using NHS chemistry. Photodegradable hydrogels were successfully prepared with amino-4-arm PEG and gelatin. Because the NHS-activated ester group is reactive with primary amines under physiological conditions,^[28] our approach enabled the preparation of photodegradable hydrogels using intact biomaterials containing amino moieties. In addition, our photocleavable crosslinker could potentially react with a variety of other natural polymers including collagen, fibronectin, and chitosan. Furthermore, some of the NHS-activated ester groups in NHS-PC-4-arm PEG could be used for chemical modification of hydrogels with functional peptides to control the cell behavior, including the adhesion, migration, maintenance, and differentiation. In contrast, methods reported in previous studies require chemical modification of the biomaterials before hydrogel preparation; for example, acrylate or methacrylate moieties are required for radical reactions,^[14,20,22–25] azides and alkynes are required for orthogonal click reactions,^[15] and dextran must be modified with acrylate for Michael-type conjugations.^[27] Compared with these other methods, our convenient approach could be advantageous for tissue-engineering research, in which cells require suitable scaffolds and microenvironments. We envision

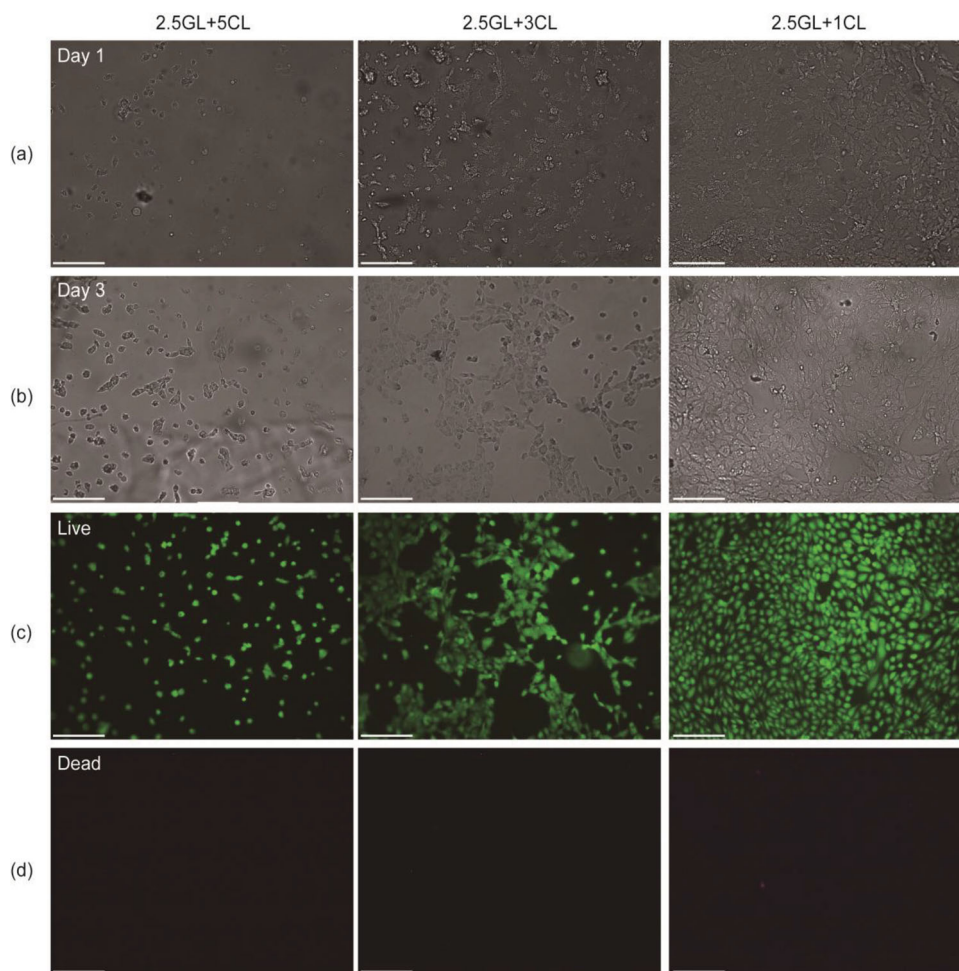


Figure 5. Microscopic phase-contrast images of HUVECs on formed photodegradable hydrogels composed of 2.5% (w/v) gelatin with 5.0% (2.5GL+5CL, first column), 3.0% (2.5GL+3CL, second column), and 1.0% (2.5GL+1CL, third column) NHS-PC-4-arm PEG after a) 1 d and b) 3 d. Cell viability was visualized by (c) live (green) and (d) dead (red) staining after 3 d of culture. Scale bars = 200 μm .

that our photocleavable crosslinker could find many applications for fabricating 3D tissue structures such as microvascular structures in the near future.

3. Conclusions

We have synthesized and characterized a photocleavable crosslinker that has the ability to form a photodegradable hydrogel upon reaction with amino-4-arm PEG or gelatin. We demonstrated that these hydrogels can be micropatterned by light irradiation through photomasks. Our new photocleavable crosslinker is potentially a versatile and convenient material for preparing photodegradable hydrogels with biocompatible polymers containing amino moieties. The degradation depth of the micropatterns could be varied by altering the light exposure. We also demonstrated that cell can be cultured on the photodegradable hydrogels with high viability and proliferation rates. The approach presented here is potentially useful for manipulating cells on hydrogels to form engineered tissue constructs, owing to its versatility with respect to chemical

composition and the simplicity of the hydrogel preparation procedure.

4. Experimental Section

Materials: All reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless specified otherwise.

Synthesis of Photocleavable NHS-PC-4-arm PEG Crosslinker: Figure 1a shows the chemical structure of our *N*-hydroxysuccinimide-terminated photocleavable crosslinker (NHS-PC-4-arm PEG). The schematic diagram of for the synthesis of the photocleavable crosslinker is shown in Figure S1 (Supporting Information). To synthesize the photocleavable crosslinker, briefly, 3-(1-piperazino)propyl-functionalized silica gel was washed with dimethyl sulfoxide (DMSO), and the washed silica gel (66 g) was then suspended in 130 mL of DMSO. 4-[4-[1-(Fmoc)ethyl]-2-methoxy-5-nitrophenoxy] butanoic acid (2.7 g; Advanced Chemtech, Louisville, KY, USA) was added to the suspension, which was stirred for 24 h at room temperature. The insoluble silica gel was removed by filtration. The DMSO solution of the resulting unprotected amino compound was concentrated under reduced pressure until the volume of the residue decreased to about 130 mL. Pentaerythritol tetra(succinimidyl carboxypentyl) polyoxyethylene (9.6 g; $M_w = 10\,998$; NOF Corp.,

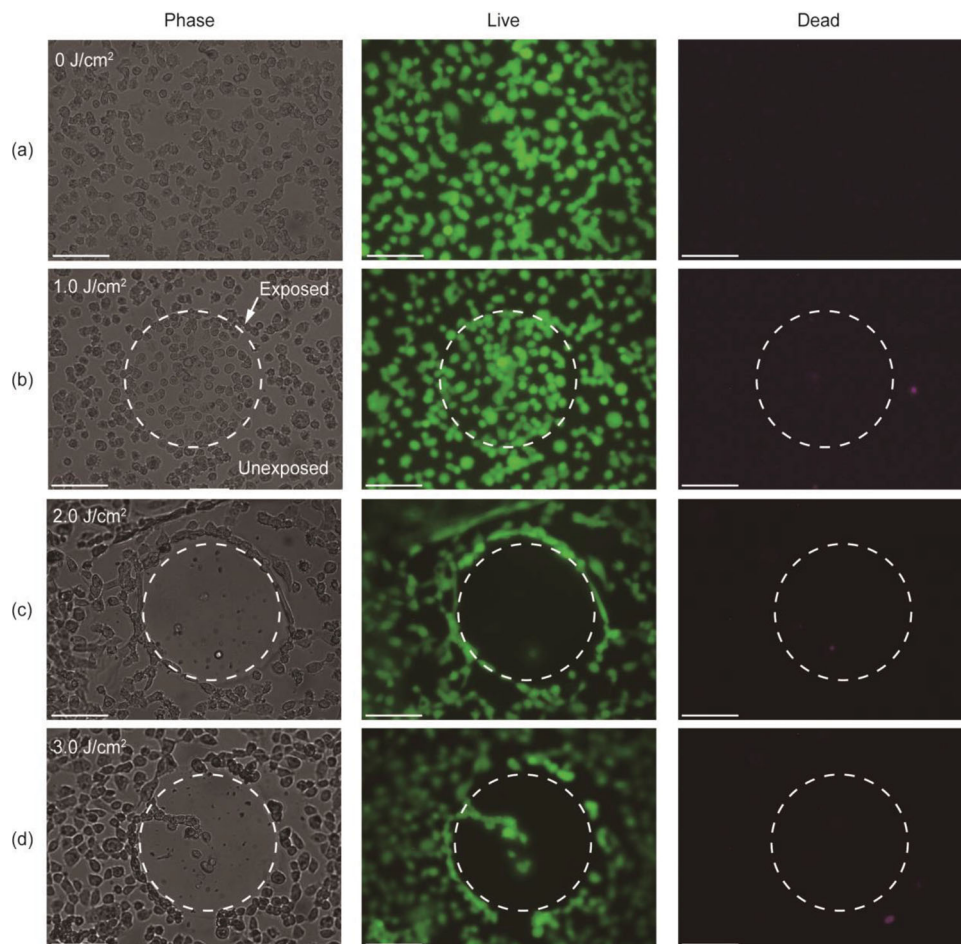


Figure 6. Effect of light exposure and hydrogel degradation on the cell viability. HUVECs cultured on the photodegradable hydrogels composed of 2.5% (w/v) gelatin with 1.0% (w/v) NHS-PC-4-arm PEG (2.5GL+1CL) for 3 d were exposed to a) 0, b) 1.0, c) 2.0, and d) 3.0 J cm⁻² light, respectively. Cell viability was visualized by live (green) and dead (red) staining after 3 h of incubation to erode the degraded polymer. Scale bars = 100 μm.

Tokyo, Japan) in tetrahydrofuran (THF) was added to the DMSO (ca. 130 mL) solution of the resulting unprotected amino compound. After the solution was stirred for 24 h at room temperature, the THF was evaporated under reduced pressure, and the residue was added dropwise into ether at 0 °C. After the reaction mixture was left for 24 h at room temperature, the precipitated carboxylic acid compound was collected by filtration and redissolved in 20 mL of THF. The precipitation was repeated two more times. The precipitate was isolated and purified with Sephadex LH-20 (ø 30 × 400 mm; GE Healthcare Japan, Tokyo, Japan) in methanol. To a THF solution of the carboxylic acid compound, 0.6 g of *N*-hydroxysuccinimide (NHS, Tokyo Chemical Industry Tokyo, Japan) and 1.0 g of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Tokyo Chemical Industry Co., Tokyo, Japan) were added, and then the mixture was stirred for 24 h at room temperature. After THF evaporation under reduced pressure, the residue was dissolved in dichloromethane. The dichloromethane phase was washed with 5% (v/v) hydrochloric acid solution and brine and then dried over magnesium sulfate anhydride, filtered, and concentrated. The residue in a small amount (ca. 20 mL) of THF was added dropwise into ether at 0 °C. After the solution was left for 24 h at room temperature, the resulting precipitate was collected by filtration. Reprecipitation was repeated two more times to afford 9.8 g of the final product (NHS-PC-4-arm PEG). The ¹H NMR spectrum was taken in deuterated chloroform (CDCl₃) at 25 °C on a Bruker Avance III 400 instrument (Bruker, Kanagawa, Japan) operated at 400 MHz (Figure S2, Supporting Information). Tetramethylsilane was used as an internal standard. Peak multiplicities are abbreviated as follows: s,

singlet; d, doublet; dd, double doublet; ddd, double double doublet; t, triplet; tt, triple triplet; m, multiplet; br, broad; dq, double quadruplet.

¹H NMR (400 MHz, CDCl₃, δ): 1.35 (m, 2H), 1.53 (d, *J* = 7.09 Hz, 3H), 1.60 (m, 4H), 2.18 (br t, *J* = 7.21 Hz, 2H), 2.28 (tt, *J* = 7.33, 5.98 Hz, 2H), 2.85 (br s, 4H), 2.88 (t, *J* = 7.33 Hz, 2H), 3.41 (s, 2H), 3.43 (t, *J* = 7.21 Hz, 2H), 3.47–3.83 (m, (O(CH₂CH₂O)_n), 3.94 (s, 3H), 4.15 (t, *J* = 5.98 Hz, 2H), 5.49 (dq, *J* = 7.57, 7.09 Hz, 1H), 6.39 (br d, *J* = 7.57 Hz, 1H), 6.91 (s, 1H), 7.56 (s, 1H).

Photocleavage of NHS-PC-4-arm PEG: The progress of the photocleavage of NHS-PC-4-arm PEG upon light exposure was estimated by measuring absorption spectra and ¹H NMR spectra. For the measurement of the absorption spectra, a 0.01% (w/v) crosslinker solution in distilled water was pipetted into a quartz cell (1 cm × 1 cm × 10 cm) and exposed to light (365 nm, 30 mW cm⁻², 10–300 s) from an ultraviolet (UV) light source (UVE-251S, San-Ei Electric, Osaka, Japan) with a combination of 350 nm long-pass and 385 nm short-pass optical filters. Absorbance spectra were measured with a UV-vis spectrometer (V560, JASCO, Tokyo, Japan). For the measurement of the ¹H NMR spectra, a 1.25% (w/v) crosslinker solution in deuterated water was pipetted into a glass NMR tube (internal diameter = 4 mm) and exposed to light (365 nm, 30 mW cm⁻², 10–300 s) from the UV light source. ¹H NMR spectra were measured on a Bruker Avance III 400 operated at 400 MHz. The degree of photocleavage was calculated by comparing the integrated peak values corresponding to the disappearance of the methyl proton signal at 1.53 ppm and the appearance of the signal at 2.15 ppm.

Preparation of Photodegradable Hydrogels: Figure 1b,c are schematic diagrams of the formation of the hydrogels by crosslinking of amine-contacting polymers. A prepolymer solution containing either 10×10^{-3} M amino-4-arm PEG ($M_w = 9617$, NOF Corp., Tokyo, Japan) or 5% (w/v) gelatin (Type A, 300 bloom from porcine skin) was prepared in a 1:1 mixture of phosphate buffered saline (PBS; Invitrogen Corp., Carlsbad, CA) and 0.3 M HEPES (Wako Pure Chemical Industries, Osaka, Japan) buffer (pH 7.0). A solution of the synthesized NHS-PC-4-arm PEG crosslinker (10×10^{-3} M, 12.1% w/v) was prepared in 10×10^{-3} M phthalate acid buffer (pH 4.0; Wako) with 140×10^{-3} M sodium chloride (NaCl, Wako). The prepolymer and crosslinker solutions were mixed in a 1:1 (v/v) ratio. Solutions containing gelatin were maintained at 37 °C, and the other solutions were kept at room temperature. Subsequently, 10–30 μ L of the mixture was pipetted onto an amino-coated glass slide (MAS coat, Matsunami Glass Corp., Osaka, Japan). NHS-PC-4-arm PEG crosslinker reacts with amino moieties on the glass slide and forms covalent bonds between the hydrogel and glass slide. The glass slide was covered with a cover slip, which was separated from the glass slide by polyethylene terephthalate (PET) films (thickness = 25 μ m) or cover slips (thickness = 150 μ m) as spacers to control the hydrogel thickness (for details, see Figure S3, Supporting Information). The mixtures were then incubated for 30 min at 37 °C to form photodegradable hydrogels. The abbreviations used to denote photodegradable hydrogels prepared from amino-4-arm PEG and gelatin under different conditions are available in Tables S1 and S2 (Supporting Information).

Rheological Characterization of Photodegradable Hydrogels: Changes in both the elastic modulus G' and viscous modulus G'' values were estimated by a rheometer (MCR-302; Anton Paar Ltd., Graz, Austria) to investigate the effect of pH on the gelation time. Following the protocol described above, the photodegradable hydrogels were prepared with amino-4-arm PEG and gelatin. Briefly, a prepolymer solution containing either 10×10^{-3} M amino-4-arm PEG or 5% (w/v) gelatin was prepared in a 1:1 (v/v) mixture of PBS and 0.3 M HEPES buffer (pH 6.6–7.0). A solution of the synthesized NHS-PC-4-arm PEG crosslinker (10×10^{-3} M, 12.1% w/v) was prepared in 10×10^{-3} M phthalate acid buffer (pH 4.0) with 140×10^{-3} M NaCl. The prepolymer and crosslinker solutions were maintained at 37 °C and mixed in a 1:1 (v/v) ratio. Immediately after the two components were mixed, 500 μ L of the mixture was put on the plate in the detector of the rheometer. The G' and G'' values at a constant frequency of 5 Hz were continuously monitored for 1 h at 37 °C with an interval time of 30 s. To evaluate the change in the pH of the solutions before and after mixing, the pH value of the solutions was measured by a pH meter (LAQUA twin B-712, HORIBA Ltd, Kyoto, Japan). Briefly, 80 μ L of the prepolymer solution containing either amino-4-arm PEG or gelatin was added to 80 μ L of the crosslinker solution containing NHS-4-arm PEG. Immediately after the two components were mixed, 150 μ L of the mixture was loaded onto the sample plate in the pH meter, and the sample was then covered with a PET film to prevent evaporation. The pH meter was then placed in the incubator at 37 °C, and the pH value was monitored.

Micropatterning of Photodegradable Hydrogels by Photolithography: Hydrogel micropatterning was performed by photolithography (for details, see Figure S3, Supporting Information). Photomasks (lines: $400 \mu\text{m} \times 5 \text{mm}$, $200 \mu\text{m} \times 5 \text{mm}$, and $100 \mu\text{m} \times 5 \text{mm}$; circles: 20–500 μ m in diameter) with different micropatterns were designed using Adobe Illustrator and printed on transparencies at a resolution of 3600 dpi. A photomask was placed on the cover slip on the photodegradable hydrogel (hydrogel thickness = 150 μ m). Subsequently, the hydrogel was placed topside-down on the sample stage and exposed to light (365 nm, 30 mW cm^{-2} , 10–300 s) from the UV light source through the photomask. To develop the photodegraded regions in the hydrogel, the sample was immersed in PBS solution for 24 h at 37 °C, during which time the degraded polymers in the irradiated area were eroded and the micropatterns were formed in the hydrogel. For visualization, patterned hydrogels prepared with amino-4-arm PEG were stained with 0.01% Coomassie Brilliant Blue (CBB, Dojindo, Kumamoto, Japan) for 1 h, and 4 \times phase contrast images of micropatterned hydrogels were then captured by an inverted microscope (IX-71, Olympus Corp., Tokyo, Japan).

Microparticle Deposition on Patterned Photodegradable Hydrogels: To evaluate the degradation depth of the micropatterned hydrogels (hydrogel thickness = 150 μ m), 3 μ m fluorescent microparticles (1% w/v solids; Duke Scientific Corp., Palo Alto, CA) were deposited on the micropatterned surface (diameter of patterned circle = 800 μ m), following the previous protocol.^[30] The surface of the hydrogel was washed with PBS, and the excess PBS on the surface was then gently removed with an air gun. A suspension of fluorescent microparticles was diluted with 10 volumes of MilliQ water (Millipore, Billerica, USA), and 30 μ L of the diluted suspension was placed onto the hydrogel and spread evenly over the micropatterned surface. The samples were then placed in a covered Petri dish humidified with a wet towel to prevent evaporation, and the particles were allowed to settle. Then, 3D 10 \times fluorescence images were obtained with a confocal laser scanning microscope (FV300, Olympus), and the degradation depth of the micropattern was measured. All experiments were repeated for four samples.

Cell Cultures: To characterize the cell behavior on the photodegradable hydrogels, HUVECs were cultured on the photodegradable hydrogels prepared with either 4-arm PEG or gelatin as a main polymer (details of the concentration of each component are available in Tables S1 and S2, Supporting Information). HUVECs were cultured in culture medium (HuMedia EG-2, Kurabo, Osaka, Japan) supplemented with 2% (v/v) fetal bovine serum, 10 ng mL^{-1} human epidermal growth factor, 1.34 $\mu\text{g mL}^{-1}$ hydrocortisone hemisuccinate, 50 $\mu\text{g mL}^{-1}$ gentamicin, 50 ng mL^{-1} amphotericin B, 5 ng mL^{-1} human basic fibroblast growth factor, and 10 $\mu\text{g mL}^{-1}$ heparin in a 5% CO_2 , 37 °C incubator. The cells were trypsinized for 5 min in a solution of 0.1% trypsin dissolved in PBS and then resuspended in culture medium at a concentration of 1.0×10^7 cells mL^{-1} . To seed cells on the hydrogels prepared with amino-4-arm PEG or gelatin, a 35 μ L droplet of the cell suspension was pipetted onto the hydrogels ($3.7 \times 3.7 \text{mm}^2$, thickness = 25 μ m) in a culture dish (35 mm^2), spread evenly over the surface, and allowed to settle. After 1 h of culture, the excess culture medium on the hydrogels was gently aspirated to remove non-adherent cells, and then 3 mL of the medium was gently added. After 1 and 3 d of culture, 4 \times phase contrast images were captured by the inverted microscope. To evaluate the cell viability after 3 d of culture, a live/dead test was performed. Cell viability was characterized by incubating the cells with live/dead assay kit (Life Technologies, Carlsbad, USA). Cells were exposed to the test solution, which was composed of 0.5 μ L of Calcein AM solution and 2.0 μ L of Ethidium homodimer-1 (EthD-1) solution in 1.0 mL of PBS, for 10 min, after which washing with PBS was carried out. After the cells were labeled with Calcein (live) and EthD-1 (dead), 10 \times phase contrast and fluorescence images were captured by the inverted microscope. To evaluate the effect of light irradiation and hydrogel degradation on cell viability, a live/dead test was also performed according to the protocol above. Cultured HUVECs on the photodegradable hydrogels composed of 2.5% (w/v) gelatin with 1.0% (w/v) NHS-PC-4-arm PEG for 3 d were exposed to light (365 nm, 125 mW cm^{-2} , 8–24 s) from the UV light source and then incubated for 3 h at 37 °C in order to erode the degraded polymer. Light irradiation of the samples was performed with a PC-controlled microprojection system (DESM-01, Engineering System, Co., Japan).^[31] For visualization, captured phase images of cells were fed into a desktop computer (Windows 7) and converted from multi-colored to grayscale images using Adobe Illustrator.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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T.T., and K.S. designed the molecular structure, experimental protocols, and performed experiments. F.Y., S.S., and T.T. processed and analyzed the data. F.Y. and S.S. wrote the paper. T.K. and A.K. supervised the research. All authors revised the manuscript and agreed on its final contents. This research was funded by the KAKENHI (24106512). The authors thank Dr. H. Minamikawa, Dr. T. Sato, and Ms. K. Morishita for technical help and helpful comment on rheological characterization.

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- [1] N. K. Inamdar, J. T. Borenstein, *Curr. Opin. Biotechnol.* **2011**, *22*, 681.
- [2] S. Khetan, J. A. Burdick, *Soft Matter* **2011**, *7*, 830.
- [3] T. Billiet, M. Vandehaute, J. Schelfhout, S. Van Vlierberghe, P. Dubruel, *Biomaterials* **2012**, *33*, 6020.
- [4] P. Zorlutuna, N. Annabi, G. Camci-Unal, M. Nikkhah, J. M. Cha, J. W. Nichol, A. Manbachi, H. Bae, S. Chen, A. Khademhosseini, *Adv. Mater.* **2012**, *24*, 1782.
- [5] B. G. Chung, K. H. Lee, A. Khademhosseini, S. H. Lee, *Lab Chip* **2012**, *12*, 45.
- [6] B. V. Slaughter, S. S. Khurshid, O. Z. Fisher, A. Khademhosseini, N. A. Peppas, *Adv. Mater.* **2009**, *21*, 3307.
- [7] Y. Du, E. Lo, S. Ali, A. Khademhosseini, *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 9522.
- [8] F. Yanagawa, H. Kaji, Y. H. Jang, H. Bae, D. Yanan, J. Fukuda, H. Qi, A. Khademhosseini, *J. Biomed. Mater. Res. A* **2011**, *97A*, 93.
- [9] R. A. Marklein, J. A. Burdick, *Soft Matter* **2010**, *6*, 136.
- [10] G. Camci-Unal, D. Cuttica, N. Annabi, D. Demarchi, A. Khademhosseini, *Biomacromolecules* **2013**, *14*, 1085.
- [11] H. Shin, B. D. Olsen, A. Khademhosseini, *Biomaterials* **2012**, *33*, 3143.
- [12] A. I. Van Den Bulcke, B. Bogdanov, N. De Rooze, E. H. Schacht, M. Cornelissen, H. Berghmans, *Biomacromolecules* **2000**, *1*, 31.
- [13] J. W. Nichol, S. T. Koshy, H. Bae, C. M. Hwang, S. Yamanlar, A. Khademhosseini, *Biomaterials* **2010**, *31*, 5536.
- [14] A. M. Kloxin, A. M. Kasko, C. N. Salinas, K. S. Anseth, *Science* **2009**, *324*, 59.
- [15] C. A. DeForest, K. S. Anseth, *Nat. Chem.* **2011**, *3*, 925.
- [16] M. P. Lutolf, *Nature* **2012**, *482*, 477.
- [17] N. Blow, *Nat. Methods* **2009**, *6*, 619.
- [18] S. M. Jay, W. M. Saltzman, *Nat. Biotechnol.* **2009**, *27*, 543.
- [19] A. M. Kloxin, M. W. Tibbitt, A. M. Kasko, J. A. Fairbairn, K. S. Anseth, *Adv. Mater.* **2010**, *22*, 61.
- [20] D. Y. Wong, D. R. Griffin, J. Reed, A. M. Kasko, *Macromolecules* **2010**, *43*, 2824.
- [21] A. M. Kloxin, J. A. Benton, K. S. Anseth, *Biomaterials* **2010**, *31*, 1.
- [22] B. Yan, J. C. Boyer, D. Habault, N. R. Branda, Y. Zhao, *J. Am. Chem. Soc.* **2012**, *134*, 16558.
- [23] S. Murayama, F. Ishizuka, K. Takagi, H. Inoda, A. Sano, T. Santa, M. Kato, *Anal. Chem.* **2012**, *84*, 1374.
- [24] D. Klinger, K. Landfester, *Soft Matter* **2011**, *7*, 1426.
- [25] B. D. Fairbanks, S. P. Singh, C. N. Bowman, K. S. Anseth, *Macromolecules* **2011**, *44*, 2444.
- [26] P. V. Chang, J. A. Prescher, E. M. Sletten, J. M. Baskin, I. A. Miller, N. J. Agard, A. Lo, C. R. Bertozzi, *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 1821.
- [27] K. Peng, I. Tomatsu, B. van den Broek, C. Cui, A. V. Korobko, J. van Noort, A. H. Meijer, H. P. Spaink, A. Kros, *Soft Matter* **2011**, *7*, 4881.
- [28] G. T. Hermanson, *Bioconjugate Techniques*, 2nd ed., Academic Press, San Diego **2008**.
- [29] J. D. Ingle, S. R. Crouch, *Spectrochemical Analysis*, Prentice Hall, Upper Saddle River **1988**.
- [30] M. J. Hancock, F. Yanagawa, Y. H. Jang, J. He, N. N. Kachouie, H. Kaji, A. Khademhosseini, *Small* **2012**, *8*, 393.
- [31] K. Kikuchi, K. Sumaru, J. Edahiro, Y. Ooshima, S. Sugiura, T. Takagi, T. Kanamori, *Biotechnol. Bioeng.* **2009**, *103*, 552.