Biomaterials Science

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Journal:	*Biomaterials Science*
Manuscript ID	BM-ART-04-2021-000585.R1
Article Type:	Paper
Date Submitted by the Author:	24-May-2021
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Photodegradable avidin-biotinylated polymer conjugate hydrogels for cell manipulation

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Protein-synthetic polymer hybrid hydrogels crosslinked via protein-ligand binding are promising materials for the three-dimensional culture of various cells; while photo-responsive hydrogels have been widely used for the spatio-temporal control of cell functions and patterning. Photo-responsive protein-polymer hybrid hydrogels are therefore attractive candidates for use in cell and artificial tissue fabrication; however, no examples combining these properties have been reported to date. Herein, a photodegradable hydrogel consisting of avidin and biotinylated polyethylene glycol (PEG) was developed as a multi-functional matrix for cell culture and sorting. A four-branched PEG with a biotinylated photocleavable group at the end of each chain was crosslinked with avidin to produce a photodegradable hydrogel. A cytokine-dependent immunocyte was successfully cultured in the hydrogel by supplying cytokine from a medium layered on the hydrogel. Additionally, the adhesion and survival of fibroblasts could be controlled by decorating the hydrogel with a biotinylated cell-adhesive peptide. Cells embedded in the hydrogels could be recovered without cell damage as a result of light-induced hydrogel degradation. Moreover, model target cells expressing red fluorescent protein were selectively liberated from a hydrogel containing cells of different colors by irradiating with targeted light. Owing to both the selective biotin-binding ability of avidin and the photocleavable properties of the synthetic polymer; the hydrogels were easy to prepare and decorate with functional molecules, provided an internal structure suitable for cell culture, and allowed light-guided cell manipulation. The hydrogels are therefore expected to contribute to various cell fabrication processes as useful cell engineering and sorting tools.

1. Introduction

Synthetic hydrogels are considered promising materials for cell and tissue engineering owing to their tailorable chemical, mechanical, biological, and stimuli-responsive properties.[1,2] Recent advances in bioscience have made it easier to obtain pluripotent cells;[3] therefore, methods to convert pluripotent cells into cells with particular functions are actively being studied.[4] In addition, methods for organizing differentiated cells into organoids and artificial tissues have been extensively studied for industrial and medical applications.[5,6] Synthetic hydrogels are applicable to various processes in cell fabrication[1,2]: they can be used as containers for cell storage,[7,8] manipulators for sorting cells,[9-12] smart matrices for tuning differentiation cues,[13,14] scaffolds for organoid generation,[15] and carriers for cell delivery systems.[16,17]

Most hydrogel-assisted processes require cells to be embedded in hydrogels that meet design criteria depending on the intended application.[1] Hydrogels for embedding cells must consist of biocompatible materials and be crosslinked through bio-orthogonal reactions. In addition, tuneable biological and physical properties such as cell adhesion, mechanical strength, and molecular permeability are desirable. Protein-synthetic polymer hybrid hydrogels crosslinked via protein-ligand binding are one of the most suitable candidates for meeting these requirements for the following reasons. First, unlike chemical crosslinkers, ligand-binding proteins are not reactive to biomolecules and thus they are highly biocompatible. In addition, protein-ligand binding has higher specificity than chemical reactions, and can achieve selective crosslinking of polymer materials, even in culture conditions where a variety of biomolecules with different reactive groups coexist.[18-22] Protein-polymer hybrid hydrogels can also be reliably decorated with additional protein factors through genetic fusion to the protein crosslinker.[21] Furthermore, compared with hydrogels based solely on proteins, synthetic polymers can provide a wider range of physicochemical properties.[23] Streptavidin is an attractive choice as a crosslinking protein because it can strongly and selectively bind biotin at four biotin-binding sites simultaneously.[19,20] Additionally, biotin can be easily introduced into both synthetic and natural molecules via the carboxylic acid moiety and various biotinylated molecules have been reported

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Electronic Supplementary Information (ESI) available: See DOI: 10.1039/x0xx00000x

and are commercially available. It has been widely reported that streptavidin in streptavidin-polymer hybrid hydrogels is not only able to crosslink the polymers, but can also display biotinylated biomolecules such as enzymes and cytokines.[20] Thus, streptavidin-polymer hybrid hydrogels are promising materials for the embedding of cells in various cell engineering processes.[22]

Photo-responsive hydrogels have recently been reported as useful tools for manipulating cells.[24] Because light irradiation can be applied with high spatio-temporal resolution, photo-responsive hydrogels often show better controllability than other stimuli-responsive hydrogels. Kloxin et al demonstrated control of the migration and differentiation of mesenchymal stem cells in 3D culture using photodegradable hydrogels based on photocleavable linkers.[25,26] The embedding of cells in photodegradable hydrogels has recently been applied to light-guided selective cell release.[9-11,17] In the applications for tuning differentiation cues, cellular adhesion was photo-controlled. In those for controlling cell migration and sorting, cells were captured by the steric restriction of the polymeric gel networks and then selectively freed at the single-cell level through local photodegradation of the hydrogels. Photodegradable protein-polymer hybrid hydrogels are expected to expand the range of applications of photodegradable hydrogels by introducing the advantageous properties of protein-polymer hybrids, however there have been no reports of photodegradable protein-polymer hybrid hydrogels to date.

In this study, we aimed to develop a photodegradable hydrogel consisting of avidin and biotinylated synthetic polymers. Egg white-derived avidin, an analog of streptavidin, was used as a biotin-binding crosslinker. Unlike streptavidin, avidin has a sugar chain and no RYD motif, so it has higher water solubility and lower non-specific cell-adhesion properties.[27,28] Poly(ethylene glycol) (PEG) was selected as the synthetic polymer component because of its biocompatibility and was biotinylated at its end group through a photocleavable linker. The biotinylated polymer was designed to form a hydrogel as a result of crosslinking through the biotin-avidin interaction, and the hydrogel could subsequently be photodegraded by light-induced removal of the biotin moiety from the polymer end (Fig. 1). Cells were successfully embedded and cultured in the hydrogel, and even adherent cells could be cultured by decorating the hydrogel with biotinylated cell adhesion peptides. Furthermore, to demonstrate the potential application of the photodegradable hydrogel for light-guided cell sorting, embedded cells were shown to be selectively released upon exposure to light.

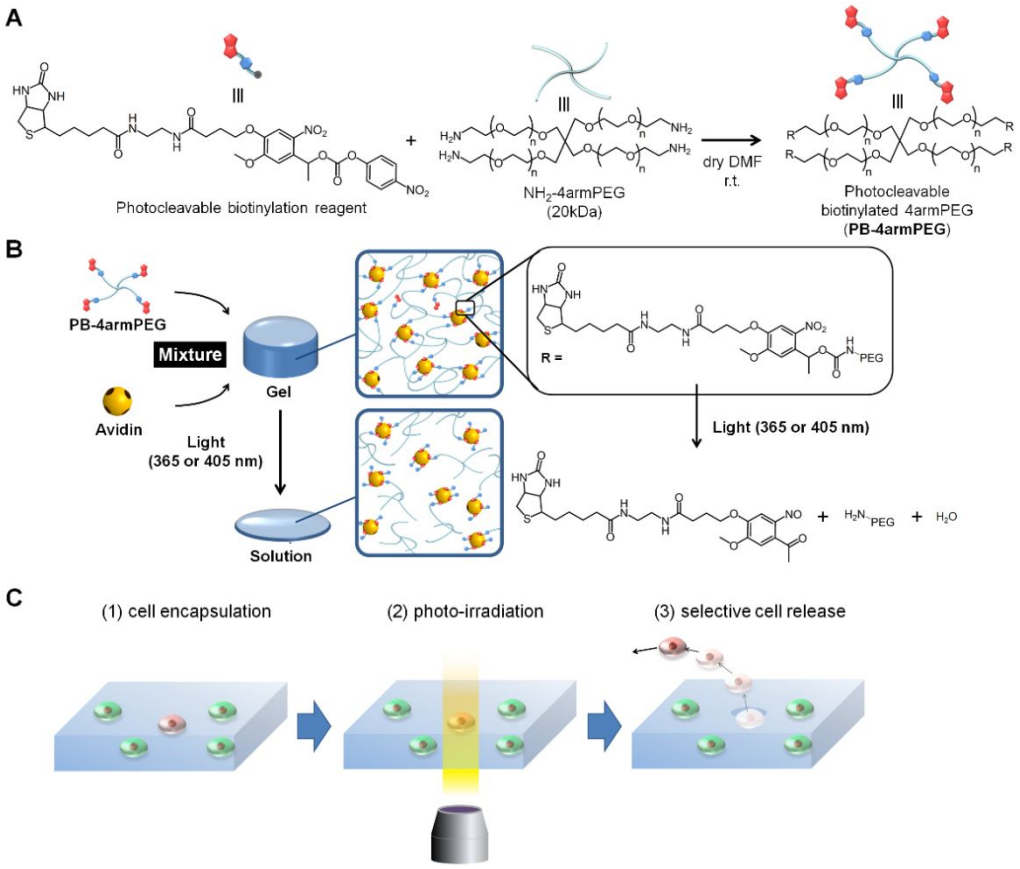

Fig. 1 Schematic illustration of photodegradable PEG-avidin hybrid hydrogels and light-guided cell release from the hydrogels. (A) Synthesis scheme and chemical structures of photocleavable biotinylated 4armPEG (**PB-4armPEG**). (B) Illustration of gelation and photodegradation of **PB-4armPEG**-avidin hydrogel. (C) Illustration of light-induced selective cell release from the hydrogel with fluorescently stained cells of two different colors embedded.

2. Materials and Methods

2.1. Materials

Unless otherwise specified, the chemicals were of analytical grade and were used without further purification. 4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy]-butyric acid, *D*-biotin (biotin), and 2-(4'-hydroxy- azobenzene) benzoic acid (HABA) were purchased from Sigma-Aldrich (St. Louis, MO). 4-Nitrophenyl chloroformate, anhydrous ethylene diamine, anhydrous *N,N'*-dimethyl formamide (DMF), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Tokyo Chemical Industry CO., LTD. (Tokyo, Japan). *N*-hydroxysuccinimide (NHS), triethylamine (TEA) and avidin from egg white were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan). Four-arm poly(ethylene glycol) (4armPEG)-amine (Mw: 20,000; Sunbright PTE-200PA) was purchased from NOF Corporation (Tokyo, Japan). Fluoresbrite® YG Microspheres (2.00 µm) were purchased from Polysciences Inc. (Warrington, PA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA). Calcein-AM and propidium iodide (PI) were purchased from Dojindo Laboratories (Kumamoto, Japan). Murine interleukin (IL)-3, RPMI-1640 medium, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL (Gaithersburg, MD). Biotinylated RGD peptide (Biotin-CGGGKEKEKEKGRGDSP)[29] was purchased from Toray Research Center, Inc. (Shiga, Japan). Enhanced green fluorescent protein (EGFP) was prepared according to our previous report.[30]

2.2. Synthesis of biotinylated macromonomers

Photocleavable biotinylated reagent (Fig. 1A) was synthesized according to our previous report.[31] Photocleavable biotinylated 4armPEG (**PB-4armPEG**) was synthesized by modifying the end groups of 4armPEG-amine (Mw: 20 kDa) with the photocleavable biotinylated reagent using the amide coupling reaction (Fig. 1A). Similarly, as a control, non-photocleavable macromonomer, biotinylated 4armPEG (**B-4armPEG**) was synthesized with biotin N-succinimidyl ester. Detailed procedures are given in the supplementary information (Scheme S1 and S2).

2.3. Hydrogel formation and light-induced degradation

The biotinylated macromonomers (**B-4armPEG** and **PB-4armPEG**) were dissolved in Dulbecco's phosphate buffered saline (PBS), and the concentration of the macromonomer solutions was determined using the colorimetric biotin assay based on the HABA-avidin complex.[32] The macromonomer solution was mixed with an avidin solution (PBS) to form a hydrogel. The final concentrations of macromonomer and avidin were 1 mM. Yellow-green fluorescent particles (diameter: 2.0 µm) were suspended in the mixture solution for staining and for single particle tracking analysis. The macromonomer-avidin solution was incubated in glass vials at room temperature for 10 min. Gelation was evaluated using a simple inversion test. The glass vial containing the solution was inverted and the solution was considered to have formed a gel when it did not fall under gravity. To induce degradation, the hydrogels in the glass vials were exposed to an ultraviolet (UV) irradiator (LAX-102, from Asahi Spectra Co., Ltd., Tokyo, Japan) equipped with a cylindrical lens and bandpass filter (wavelength: 365 ± 5 nm, 2.5 mW/cm^2). After light exposure, degradation was determined using the inversion test described above.

2.4. Evaluation of gel network formation

Formation of a gel network for trapping micro-sized objects was evaluated by single particle tracking analysis.[33,34] A drop of the **PB-4armPEG**-avidin solution containing fluorescent particles was incubated on a glass-bottom dish at room temperature for 10 min. A part of the hydrogel was irradiated with a diode laser (405 nm, < 30 mW/cm^2) for 2 min, using the region of interest (ROI) mode of a confocal laser scanning microscope (CLSM) (LSM 510 META, Carl Zeiss, Germany). The Brownian motion of the fluorescent particles in the irradiated and non-irradiated area was observed with CLSM as described in our previous report.[33] The velocity of the microparticle motion was calculated with the Particle Track and Analysis (PTA ver 1.2) plug-in of ImageJ (NIH, Bethesda, MD).

2.5. Oscillatory rheology measurements

The oscillatory shear rheological properties, that is, the storage elastic modulus (G') and the loss elastic modulus (G''), were measured by sweeping the frequency from 100 Hz to 0.01 Hz at 25 °C with an Anton Paar Physica MCR 301 rheometer (Anton Paar, Graz, Austria).[35] **PB-4armPEG** and avidin were dissolved in PBS at a concentration of 1 mM and a cylindrical hydrogel with a thickness of 0.3 mm was prepared by placing the **PB-4armPEG**-avidin solution in a mold at room temperature for 10 min. Parallel-plate geometry was employed using a 25 mm diameter plate with a constant deformation of 1%.

2.6. Cell embedding and culture

A **PB-4armPEG** solution in PBS was added to a glass-bottom dish (35 mm), then murine IL-3-dependent pro-B (BaF3) cells suspended in avidin PBS solution were added dropwise to the **PB-4armPEG** solution. The solutions were immediately mixed with a pipette to give a **PB-4armPEG**-avidin solution (final concentration of both: 1 mM, total volume: 30 µL) containing BaF3 cells (final cell density: 1×10^6 cells/mL). After allowing to gel for 10 min, the hydrogel with embedded cells was rinsed with PBS (1 mL). For cell culture in the hydrogel, culture medium (RPMI-1640 medium supplemented with 1 ng/mL IL-3 and 10% FBS, volume: 2.5 mL) was layered on the hydrogel and then the sample was incubated at 37 °C under 5% CO_2 for 2 days. To fluorescently stain the live and dead cells in the hydrogel before and after incubation, a staining solution (2 µM calcein AM and 1.5 µM PI; volume: 1 mL) was added to the dish and the sample was incubated at 37 °C for 1 h. After washing with PBS, the embedded cells were observed with CLSM by setting the

observation focus higher than the bottom of dishes. To quantify the cell viability following 3D culture for 2 days, the hydrogel containing embedded cells was exposed to light (2.0 J/cm^2) to induce photodegradation as described above. After staining the dead cells with PI, the dead cell ratio of the recovered cells was analyzed using a flow cytometer equipped with an argon laser (FACS-Calibur, Becton-Dickinson, USA).

2.7. Functionalization of the hydrogel with biotinylated peptides

Murine embryonic fibroblasts (NIH3T3 cells) were suspended in **PB-4armPEG** solution, and then the cell suspension was poured into a glass-bottom dish. Biotinylated RGD peptide was dissolved in an avidin solution and incubated to allow the RGD peptide to conjugate avidin. The RGD peptide-avidin complex solution was then added dropwise to the cell suspension containing **PB-4armPEG** on the dish to obtain an RGD peptide-decorated **PB-4armPEG**-avidin solution (**PB-4armPEG**: 1 mM, avidin: 1 mM, biotinylated RGD peptide: from 0 to 1 mM, total volume: 30 μL) containing NIH3T3 cells (1×10^6 cells/mL). After gelation and rinsing, culture medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS) was added to the hydrogel, and the system was cultured for 3 days. After staining live and dead cells, the embedded cells were observed by CLSM as described in the section 2.6.

2.8. Light-guided cell release

A **PB-4armPEG**-avidin solution containing a mixture of BaF3 cells expressing enhanced green fluorescent protein (EGFP) and Kusabira-Orange (KO) (EGFP-BaF3 and KO-BaF3 cells, respectively) (EGFP-BaF3 : KO-BaF3 = 4:1, final cell density: 1×10^6 cells/mL) was prepared on a glass slide using a procedure similar to that described in the section 2.6. A concave mold made of PDMS was pressed against a droplet of cell suspension and after gelation for 5 min the mold was removed and the thin hydrogel (thickness: 100 μm) with embedded cells was washed with PBS. The hydrogel was surrounded by a 2-mm-thick PDMS spacer, immersed in the culture medium, and covered with a cover glass to prevent it drying out. After confocal microscopy observation, the embedded KO-BaF3 cells were selectively exposed to light (405 nm, 0.5 mW) for 4 min using CLSM.[10] The light-exposed cells were released from the hydrogel by rinsing with the culture medium: after removing the spacer and cover glass, the glass slide with the hydrogel was repeatedly dipped vertically into medium in a 50-mL tube and pulled up (five times) for a uniform and reproducible rinse. Selective cell release was evaluated with CLSM by observing the same area before and after rinsing. The residual cell rate was determined in each three trial, and the mean of the values was calculated.

3. Results and discussion

3.1. Basic properties of PEG-avidin hybrid hydrogels

We first investigated the hydrogel formation of biotinylated PEG through crosslinking with avidin. In a preliminary trial, 4arm-branched PEG with biotinylated ends were used in gelation tests. These biotinylated PEGs were mixed with avidin at a ratio such that the biotinylated polymer ends were equimolar to the biotin-binding sites of avidin, based on previous reports on hydrogels consisting of two reactive macromonomers.[36] Mixing at high concentrations resulted in gelation that was too fast for uniform mixing. The maximum PEG concentration at which the two components could be manually mixed with a pipette into a uniform solution under our experimental conditions was 1 mM. Therefore, the gelation test was performed at a PEG concentration of 1 mM. Inversion tests quickly confirmed that the biotinylated PEGs formed hydrogels in the presence of avidin. Furthermore, the hydrogels were stable against the additional layering of PBS and even FBS-supplemented culture medium on the top of them in the glass vials (Fig. S1).

The basic properties required for cell embedding and manipulation were then examined. First, the permeability of a matrix to protein factors such as chemokines and cytokines is critical for the growth and differentiation of the embedded cells. To investigate the permeability of the **B-4armPEG**-avidin hydrogel to proteins, we monitored the release of fluorescent proteins embedded in the hydrogel into a solution layer on the hydrogel. The time-course change in fluorescence intensity of the solution confirmed that the fluorescent proteins were transferred from the hydrogel to the solution, likely according to the concentration gradient (Fig. S2). This result indicates that proteins can diffuse through the gel networks of the hybrid gels and suggests that the reverse supply of protein factors from the medium into the gel is also possible. Second, for cell manipulation in 3D cell culture and cell sorting, gels require the ability to capture cells. To evaluate the ability of the gels to capture micro-sized objects, fluorescent microparticles with a diameter of 2 μm were suspended in a mixture of **B-4armPEG** and avidin solutions. Observation under a microscope showed that the Brownian motion of the microparticles stopped completely after gel formation (Movie S1). This result strongly suggested that the hybrid gel would be able to capture cells larger than 2 μm via the formation of a gel network.[33,34] Thus, it is believed that the mesh of the hydrogel is sufficiently large compared with the size of proteins and sufficiently small compared with the size of general mammalian cells. It was therefore confirmed that the 4armPEG-based hybrid hydrogel meets the basic requirements for use in cell culture and manipulation.

3.2. Hydrogel formation and photodegradation

Photodegradable hybrid hydrogels were developed using biotinylated 4-armPEG derivatives and avidin. To introduce photodegradability, a photocleavable methyl-6-nitroveratryloxycarbonyl (MeNVoc) linker was inserted between biotin and the end of the 4armPEG chains, producing **PB-4armPEG** (Fig. 1B). The gelation of a mixture of **PB-4armPEG**

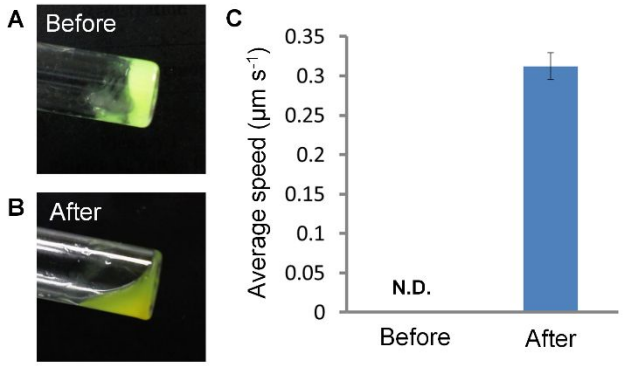

Fig. 2. Photographs and particle tracking microrheology of photodegradable PEG-avidin hybrid hydrogels with yellow fluorescent microparticles before and after exposure to light. (A) Photographs of the mixture of **PB-4armPEG** and avidin solutions before and (B) after light exposure (365 nm, 10 J/cm^2). (C) Velocities of fluorescent microparticles in the light-irradiated and non-irradiated area of the hydrogel. The values are means ± standard error (in the two trials, total seven and eight particles were observed in the irradiated and non-irradiated area, respectively).

and avidin solutions was quickly checked using an inversion test (Fig. 2A). The **PB-4armPEG**-avidin mixture in PBS was also examined by dynamic viscoelasticity measurement. As shown in Fig. 3, the storage modulus (*G*') was higher than the loss modulus (*G*") over the entire frequency range, indicating that the elastic behavior observed in the gel state is predominant. Additionally, the viscoelasticity of the **PB-4armPEG**-avidin mixture in both PBS and the culture medium were almost the same (Fig. S4). These results indicate that the hydrogels formed were similar, even when formed in medium supplemented with FBS. Subsequently, the photodegradation of the **PB-4armPEG**-avidin hydrogel was evaluated by exposure to light. The MeNVoc group has been reported to be cleaved by exposure to light at wavelengths from 350 to 420 nm.[37] Therefore, the hydrogel was exposed to light with a wavelength of 365 nm. Following exposure at 10 J/cm^2, the inversion test confirmed that the hydrogel has dissolved (Fig. 2B). In the photodegraded product, photocleavage of the MeNVoc linker of **PB-4armPEG** was confirmed from the spectral change in UV/vis absorbance spectra (Fig. S3). This result suggests that the present photodegradation of the hydrogel occurred through

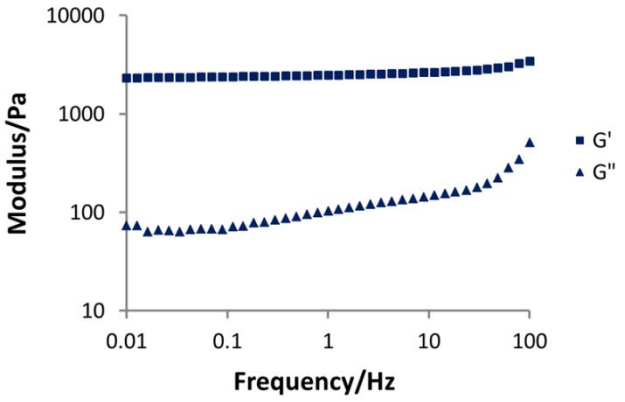

Fig. 3. Dynamic viscoelasticity measurement of photodegradable PEG-avidin hybrid hydrogels (*G*': storage modulus, *G*": loss modulus).

photocleavage of the MeNVoc linker as designed. Thus, it was macroscopically confirmed that a photodegradable PEG-avidin hybrid hydrogel could be prepared according to our molecular design.

To microscopically examine the photodegradation of the hydrogels, the motion of fluorescent microparticles captured by the gel network was observed using confocal microscopy.[33,34] The lower-right quadrant of the observation frame was selectively exposed to laser light with a wavelength of 405 nm. Light exposure triggered the motion of microparticles in the light-exposed region (Movie S2). This result clearly shows that the captured microparticles were liberated in response to light, likely owing to the photodegradation of the gel network. Particle tracking analysis determined that the average speed of the microparticles following exposure to light was 0.31 μm/s (Fig. 2C), which is slower than that of particles in a polymer-free buffer solution (0.50 μm/s). This result suggests that macromonomers or oligomers consisting of 4armPEG and avidin are generated by local photodegradation of the gel structures in the light-exposed region, increasing the viscosity of the solution. Thus, the photodegradability of the hybrid gel was also confirmed microscopically. Furthermore, microscopy observation demonstrated that the motion of micrometer-sized objects in the hydrogel could be selectively photo-controlled.

3.3. Hydrogel embedding and release of cells

A model cell was embedded in the photodegradable PEG-avidin hybrid hydrogel. To evaluate the cytocompatibility of the hydrogel, we selected a nonadherent immunocyte, BaF3, because it does not require adhesion factors in the hydrogel. BaF3 cell requires only the soluble factor IL3 for survival. Therefore, after embedding the cells, the hydrogel was incubated with culture medium containing a biologically relevant concentration of IL3. First, immediately after embedding the cells, it was observed that the green fluorescently stained cells were monodispersed in the 3D hydrogel (Fig. 4A–D). In the differential interference contrast (DIC) image, the cells appeared to be surrounded by a lumpy gel structure. These images suggest that the cells were captured in the gel networks. Furthermore, live/dead staining showed that all of the observed cells were fluorescently stained green with calcein, and no red PI fluorescence for dead cells was observed. This result indicates that both the materials and the gelation process of the hybrid gel are highly cytocompatible. Next, after culture for 2 days, clusters consisting of a few or several cells were observed in some places (Fig. 4E–H). This suggests that some embedded cells may have divided by pushing away the surrounding gel structure. Moreover, as before, no red-stained cells were observed in the hydrogel, indicating that the model cells could be cultured in the hydrogel without cell death.

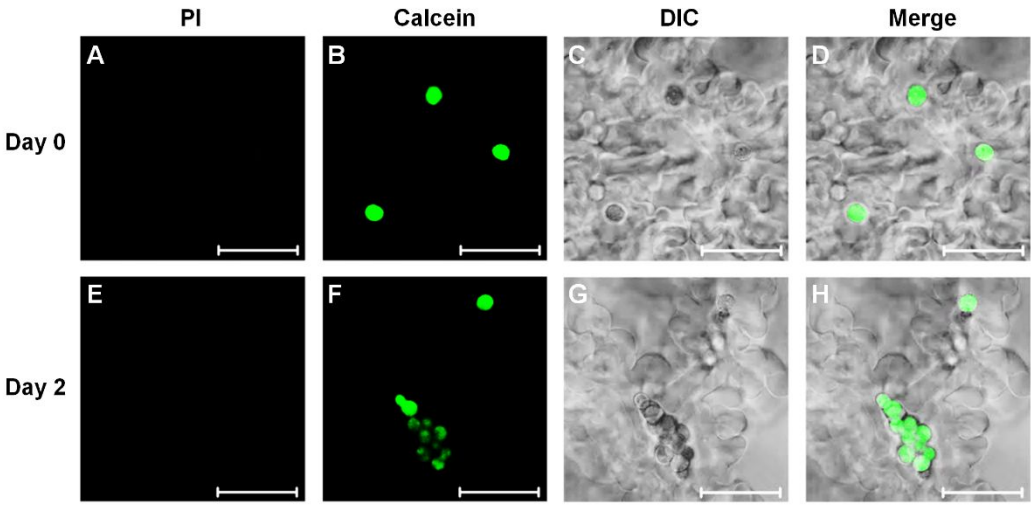

Fig. 4 Confocal microscopy images of the cells embedded in photodegradable PEG-avidin hybrid hydrogels. (A–D) BaF3 cells were embedded and fluorescently stained with PI (red) and calcein (green) immediately after embedding and (E–F) after culture for 2 days. (A,E) Red fluorescence, (B,F) green fluorescence, (C,G) differential interference contrast (DIC) images, and (D,H) their merged images. Scale bars: 50 μm.

The embedded cells were recovered by hydrogel photodegradation. The hydrogel with embedded cells was exposed to light at 2 J/cm^2, and the solution of degraded hydrogel containing the cells was collected. The cell viability was then analyzed by flow cytometry. The viability of the recovered cells was 94% and was not markedly different to that of non-treated cells (98%) (Fig. S5). These results confirmed that embedding cells in the photodegradable hydrogel and using light to release them, did not influence the viability of the embedded cells.

3.4. Adherent cell culture in peptide-decorated hydrogels

To demonstrate facile functionalization with biotinylated synthetic molecules, the photodegradable hybrid hydrogel was modified with a biotinylated cell-adhesive peptide for adherent cell culture. Adherent cells have an integrin family of cell-adhesion receptors on the cell surface that interact with the extracellular matrix to exert essential cellular functions such as cell elongation, differentiation, and survival.[38] Therefore, synthetic hydrogels are often modified with peptides including the RGDS sequence derived from fibronectin to facilitate cell adhesion through binding to integrins.[39-41] In this study, we added an *N*-terminal biotinylated peptide including the RGDS sequence (biotin-RGDS peptide) to the hydrogel. Before mixing with **PB-4armPEG** solution, the RGDS peptide was added to the avidin solution at various final concentrations in the range 125–1000 μM. The final concentration of avidin was 1 mM. Accordingly, the molar ratio of the peptide to avidin was from 0.125 to 1; therefore, up to one of the four biotin-binding sites of avidin was used for peptide modification, and the residual binding sites were used to crosslink **PB-4armPEG**. Mouse fibroblast NIH3T3 cells were used as model adherent cells because of their high adhesion to RGDS-modified surfaces.[41,42]

The morphology of the cells was examined after culture on the surface of the hydrogel. It has previously been reported that morphological differences in NIH3T3 cells on various surfaces are clearly detected in a two-dimensional culture system.[41] Therefore, in the preliminary trial, to certainly confirm the morphological differences, cells were not embedded in the hydrogel but seeded on a thin hydrogel with a flat molded surface. Confocal microscopy showed that cells cultured on the hydrogel decorated with biotin-RGDS peptide at a concentration of 250 μM, were more elongated than those cultured on the non-decorated and biotin-decorated hydrogels (Fig. S6). The morphological difference derived from the decoration of the peptide could be quantitatively confirmed by calculating the mean aspect ratio of the cells by image analysis (Fig. S6E). Thus, the successful functionalization with biotin-RGDS was preliminarily confirmed by the two-dimensional culture of adherent cells on the hydrogel.

Figure 5 shows confocal microscopy images of cells embedded in hydrogels decorated with a biotin-RGDS peptide after culture for 2 days. Above peptide concentrations of 500 μM (Fig. 5A and B), cells were clearly observed to elongate in the hydrogels, while below 125 μM, cells aggregated into spheroid-like structures without elongation (Fig. 5D and E). This concentration-dependent morphological difference indicates that the embedded cells adhered to the surrounding gel structures as the modified biotin-RGDS peptide density increased. Moreover, red-stained apoptotic cells were only clearly observed in the hydrogel without biotin-RGDS peptide modification (0 μM, Fig. 5E). This result indicates that the signals essential for cell survival were induced in the embedded cells through interaction with the decorated biotin-RGDS peptide. The concentration of biotinylated cell-adhesive peptides used to decorate the gel could therefore be used to control the morphology and survival of adherent cells within the hydrogel. Therefore, the proliferation, differentiation, and fate of other cells in the hybrid gels are potentially controllable via

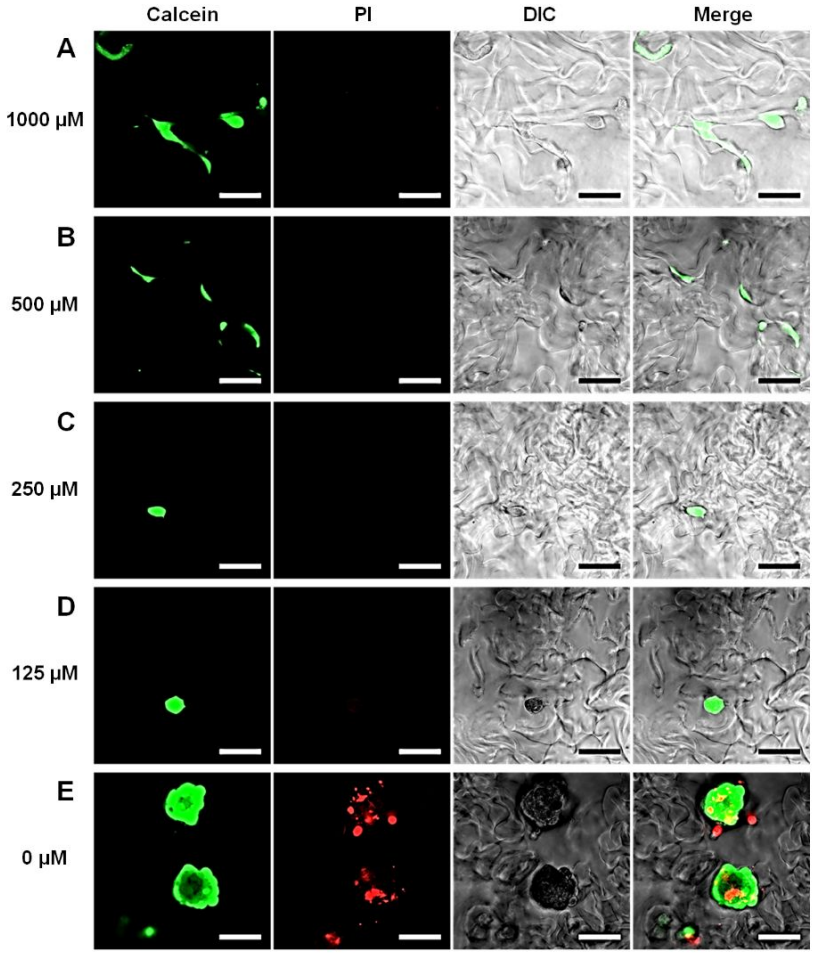

Fig. 5 Confocal microscopy images of adherent cells embedded in RGD peptide-functionalized photodegradable PEG-avidin hybrid hydrogels. NIH3T3 cells were embedded and fluorescently stained with calcein (green) and PI (red). Fluorescence and DIC images were obtained and the images were merged image. The hybrid hydrogels were decorated with biotinylated RGDS peptide at concentrations of (A) 1000, (B) 500, (C) 250, (D) 125, and (E) 0 μL. Scale bars: 50 μm.

presentation of a wide variety of peptides for cell-surface receptors. Such peptide-oriented cell control has been achieved in synthetic hydrogels by chemical modification of the materials.[43] Compared with these reported materials, our hybrid gels could potentially allow for more facile tuning of the culture environments by in situ combinatorial addition of biotinylated peptide candidates at various concentrations and ratios, without the need to synthesize new materials on a condition-by-condition basis.

3.5. Light-guided cell release

Finally, we conducted light-guided cell release from the photodegradable hydrogels to investigate their potential application for cell sorting. The requirements of the photodegradable hydrogels for cell sorting are as follows: (1) It is necessary to capture cells securely, without non-specific liberation at locations not exposed to light; (2) It is necessary to liberate the embedded cells through local degradation in response to a non-cytotoxic dose of light. In terms of the cytotoxicity of light, the previous experiment confirmed that the embedded cells could be recovered from the hydrogel in a live state by light irradiation (Fig. S5). Accordingly, we performed an experiment to confirm selective cell release using two kinds of BaF3 cells expressing fluorescent proteins of different colors. A mixture of these cells was embedded in a photodegradable hydrogel, and then only cells with a particular color fluorescence were targeted for photo-release. Green fluorescent EGFP-BaF3 and red fluorescent KO-BaF3 cells were embedded in a thin hydrogel layer. Before exposure to light, confocal microscopy showed that both fluorescent cell types were embedded (Fig. 6A, left). The red fluorescent cells were then selectively irradiated with a single cell-sized light spot under microscopy observation, and the hydrogel layer on the glass slide was repeatedly rinsed. After rinsing, only green fluorescent cells were found to remain in the same location as before light exposure (Fig. 6A, right). Moreover, the light-irradiated spots where the red fluorescent cells had been before light exposure, had holes and appeared black. This result indicates that the KO-BaF3 cells were released as a result of light-induced local degradation of the hydrogel. The quantitative image analysis shows that the residual rates of

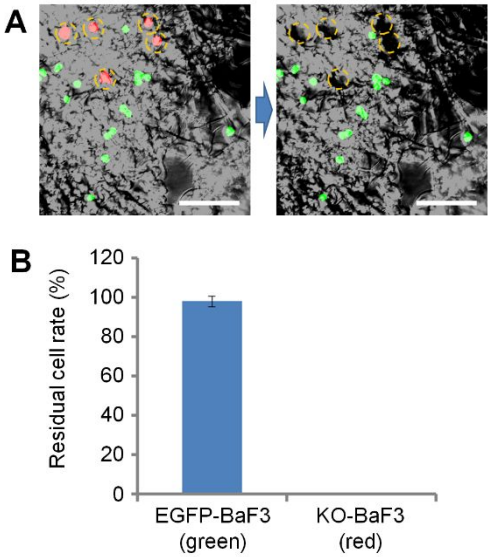

Fig. 6. Light-induced selective cell release from photodegradable PEG-avidin hybrid hydrogels. (A) Confocal microscopy images of the embedded cells; EGFP-expressing Ba/F3(EGFP-BaF3, green) and KO-expressing Ba/F3(KO-BaF3, red) cells (*left*) before and (*right*) after light irradiation. Light-irradiated areas are shown in orange dashed circles. Scale bars: 100 µm. (B) Residual rate of each type of cells after light irradiation (n = 3, mean ± S.E.).

green and red fluorescent cells in the hydrogel were approximately 100 % and 0 %, respectively (Fig. 6B). This result confirmed that the target cells were selectively released from the hydrogel, without non-specific cell release, by exposure to a spot of light. Thus, the photodegradable hydrogel is a promising material for image-based light-guided cell sorting.

4. Conclusion

Photodegradable protein-synthetic polymer hybrid hydrogels were developed by copolymerization of a photocleavable biotinylated four-armed PEG with avidin through biotin-avidin binding. Rapid and stable gelation was achieved by simply mixing the two solutions of biotinylated polymer and avidin under physiological conditions, even in the presence of serum-supplemented culture medium. When exposed to light, the hybrid hydrogel lost its gel network and was photodegraded. Owing to the non-cytotoxicity of both the material and the gelation reaction, cells could be embedded and cultured in the hydrogel. By decorating with biotinylated cell adhesive RGDS peptide, fibroblasts could be adhered to the surrounding gel structures, and were found to elongate and could be cultured without dying. Furthermore, the embedded cells could be released from the hydrogel by exposure to a non-cytotoxic dose of light, and selective liberation of the target cells could be achieved in a light-guided manner. Thus, the hydrogel presents an internal structure suitable for both the culture and control of the embedded cells, allowing the soluble factors necessary for cell survival to diffuse freely, while the cells were tightly captured by the steric restrictions of the enclosed polymeric structures. Additionally, cell behavior could be controlled by incorporating biotinylated peptides into the hydrogel. As biotinylation is easy and applicable to a wide variety of molecules such as protein ligands, DNA aptamers, and synthetic sensors, there is the potential to introduce such molecules into the hydrogel to control differentiation, sensitize to specific receptors, and monitor cellular secretions, respectively. Moreover, further systematic study of the molecular weight, branch structure and concentration of polymeric macromonomer may lead to control the mechanical properties and photo-responsibility in a wide range. Therefore, cells embedded cells in the hydrogel could be manipulated and evaluated through multifaceted approaches. Furthermore, the specifically engineered and identified cells could subsequently be recovered by light-induced release in a selective and high-throughput manner. Thus, the photodegradable hydrogels are promising tools for cell fabrication and screening in various research fields and will contribute to broadening the potential of protein-polymer hybrid hydrogel systems through photodegradability.

Author Contributions

S.Y. and T.N. conceptualized the study. S.Y. and K.M. designed the experiments. N.O., K. M. and S.Y. performed the experiments. N.O. and K.M. analyzed the data. N.O. developed reagents. S.Y., K.M. and T.N. administrated and supervised the study. S.Y. and N.O. wrote the original draft.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Murine pro-B cell line BaF3 expressing fluorescent protein was kindly donated by Prof. Masahiro Kawahara of the National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN). Prof. Takamasa Sakai of The University of Tokyo kindly lent a rheometer and advised on the dynamic viscoelasticity measurement. This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan, Grant-in-Aid for Young Scientists (A) 24686094, and by the Japan Science and Technology Agency (JST), PRESTO 16815021 and MIRAI program 19217334. We thank Sarah Dodds, PhD, from Edanz Group (https://en-author-services.edanz.com/ac) for editing a draft of this manuscript.

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