Two-Phase Bioreactor System for Cell-Laden Hydrogel Assembly

Muhammad Gulfam, Jong Min Lee, and Bong Geun Chung
Dept. of Bionano Engineering, Hanyang University, Ansan 426-791, Korea

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Bottom-up approach is a potentially useful tool for hydrogel assembly of cell-laden individual building blocks. In this article, we assembled individual building blocks of photocrosslinkable microgels in a rapid and controlled manner. Individual building blocks of poly(ethylene glycol) (PEG) microgels with square and hexagonal shapes were fabricated by using a photolithography technique. Individual building blocks of PEG microgels were assembled on a hydrophobic mineral oil phase in a bioreactor with a magnetic stirrer. The hydrophobic mineral oil minimized the surface free energy to assemble hydrophilic PEG microgels on a two-phase oil-aqueous solution interface. We used the hydrophobic effect as a driving force for the hydrogel assembly. Various types of the hydrogel assembly were generated by controlling the stirring rate. As stirring speed increased, the percentage of linear, branched, and closely packed hydrogel assembly was increased. However, the percentage of random assembly was reduced by increasing stirring rate. The stirring time also played an important role in controlling the types of hydrogel assembly. The percentage of linear, branched, and closely packed hydrogel assembly was improved by increasing stirring time. Therefore, we performed directed cell-laden hydrogel assembly using a two-phase bioreactor system and optimized the stirring rate and time to regulate the desired types of hydrogel assembly. Furthermore, we analyzed cell viability of hydrogel linear assembly with square shapes, showing highly viable even after secondary photocrosslinking reaction. This bioreactor system-based hydrogel assembly could be a potentially powerful approach for creating tissue microarchitectures in a three-dimensional manner. © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.,* 27: 466–472, 2011
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Introduction

Hydrogels that can create three-dimensional (3D) tissue architectures in vitro are of great interest in various tissue engineering and biological applications.[1,2] Hydrogel is an attractive biomaterial for controlling the shape and structure of 3D tissue microarchitectures. Hydrogel that shows biocompatible, biodegradable, and hydrophilic 3D networks has been extensively used as a scaffold, because its physical, mechanical, and biological properties are similar to natural extracellular matrix (ECM).[3–5] To create tissue microarchitectures in vitro and regulate cell-extracellular microenvironment interactions, the mechanical stiffness and porosity of natural and synthetic hydrogels can also be controlled. Thus, the hydrogel-based assembly is a potentially powerful approach to mimic complex native tissue constructs, because most tissues generally consist of modular functional microscale tissue units, such as sinusoid of liver tissue.[6]

To control the size and shape of microgels that can mimic the 3D tissue constructs, various microscale technologies (i.e., photolithography, micromolding, agitation on two-phase interface system, and microfluidics) have been previously developed.[7–13] To date, top-down and bottom-up approach has been widely used for microscale tissue engineering applications.[7] In particular, the bottom-up approach has been increasingly used for creating cell-laden hydrogel assembly that has the potential to create engineered tissue constructs. For example, individual building blocks of cell-laden microgels have been previously generated from poly(dimethylsiloxane) (PDMS) micropatterned substrates to generate checkerboard patterned assembly.[8] Although cell-laden microgels were created by using the micromolding technique, the patterning of hydrogel assembly is not easily controllable and scalable. Hydrogels have also been directly assembled by using a mechanical agitation on a two-phase oil-aqueous solution.[9] Despite the potential to direct hydrogel assembly using a mechanical agitation, this approach has some limitations, such as the inability to precisely control assembly types. It is probably due to a pipette tip-based manual mechanical agitation. The hydrogel assembly is generated by manually agitating a pipette tip; however, the assembly types may be changed by hand skills of the users. The microcontact printing technique has been used to assemble cell-laden microgels.[12] The microcontact printing technique enabled the control of hydrophilic regions and octadecyltrichlorosilane (OTS)-linked hydrophobic regions. It was revealed that the hydrogel assembly was only confined within hydrophilic regions. Recently, the interface-directed self-assembly technique has been developed to

Correspondence concerning this article should be addressed to B. G. Chung at bchung@hanyang.ac.kr.

create tissue-like architectures.[13] The tightly packed and ordered cell-laden hydrogel assembly was generated at a liquid–air interface by using a high-density hydrophobic solution. From this liquid–air interface approach, centimeter-scale tissue constructs containing a number of lock-and-key complex individual building blocks were assembled to co-culture the human hepatocellular liver carcinoma cells (HepG2) and fibroblast cells. In addition to previous experimental approaches of the hydrogel assembly, computational modeling of cell-laden hydrogel assembly has been previously studied for shape-controlled hydrogel assembly.[14] For the stochastic computational modeling, a two-dimensional (2D) coarse-grained off-lattice Monte Carlo model with Lennard–Jones-type potential was used. It was revealed that hydrogel assembling force was surface tension between a hydrophilic PEG gel and hydrophobic mineral oil phase, and simulations for rectangular units were corresponded to the experimental results of previous manual agitation-based hydrogel assembly system.[9]

Microfluidic devices have been used to create individual microgel building blocks for the hydrogel assembly.[10,15,16] For instance, the stop-flow lithography technique has been used to create individual building blocks of cell-laden microgels, showing various shapes (i.e., square, triangle, and circle) of microgels.[10] The prepolymer solution containing cell suspension was infused through the microchannel, and cell-laden poly(ethylene glycol) (PEG) microgels were subsequently photocrosslinked during the stop-flow process. Although shape-controlled cell-laden individual microgel building blocks were generated in a high-throughput manner, hydrogel assembly was not studied in a microfluidic device. 3D monolithic microstructures have also been developed by using a photolithography technique in a microfluidic device.[15] Individual building blocks of PEG microgels were assembled to make chain-like patterns. Although each microgel was easily aligned, this approach has the lack of creating thicker tissue structures. Recently, the railed microfluidic channels have been used to generate the fluidic-based complex self-assembly in a well-defined microenvironment.[16] Each microtrain containing male and female latching beams was self-assembled by using the fluidic flow. Although various self-assembled microstructures were generated by using this railed microfluidic device, some limitations are still remained, such as the inability to harvest assembled microstructures from PDMS microchannels. Given these advantages of previous bottom-up approaches, individual building blocks of cell-laden microgels containing various sizes and shapes could be assembled for creating the tissue-like constructs in a rapid and controlled manner.

In this article, we used the bioreactor system and two-phase oil-aqueous interface between hydrophilic PEG and hydrophobic mineral oil that can minimize the surface free energy as previous approach has been described.[9] For the cell-laden hydrogel assembly, photocrosslinkable individual building blocks of microgels were stirred on the hydrophobic medium using a bioreactor with a magnetic stirrer. We hypothesized that the stirring rate and time would generate different types (linear, branched, and random) of cell-laden hydrogel assemblies with square shapes. We also demonstrated that stirring speed and time enabled the control of the closely packed and random hydrogel assembly with hexagonal-shaped individual building blocks. Furthermore, the viability of the cells encapsulated within the hydrogel square assembly was analyzed by using a live/dead assay, showing highly viable even after secondary photocrosslinking reaction. Therefore, this bioreactor system-based hydrogel assembly could be a potentially powerful approach for creating 3D tissue microarchitectures in vitro.

Materials and Methods

Fabrication of individual building blocks of PEG microgels

Poly(ethylene glycol) dimethacrylate (MW 700, Sigma) was mixed with 1% photoinitiator (Irgacure 2959, CIBA Chemicals) for the photopolymerization. A photomask thin film with square shape (500 μm $\times$ 500 μm) and hexagonal shape (500 μm in length) was designed by using AutoCAD. Two glass spacers were placed on a glass slide, and a solution containing 40 μL of photocrosslinkable prepolymer solution and photoinitiator was dropped on a glass slide. The upper glass slide was placed on top of prepolymer solution to form a uniformly distributed layer of prepolymer solution. A photomask thin film was then placed on upper glass slide. The prepolymer solution was photocrosslinked by using a UV light (Omnicure, 6.00 mW/cm^2) for 2.5 s (Figure 1). Both glass slides were treated with OTS (FW 387.94, Aldrich) solution before UV exposure for making hydrophobic surface of glass slides.

Hydrogel assembly in a bioreactor

The top glass slide and glass spacers were removed after the photolithography process. Individual building blocks of PEG microgels were transferred into a Petri dish containing 10 mL mineral oil (Aldrich Chemical Company, USA). Individual building blocks of hydrophilic PEG microgels were assembled on hydrophobic mineral oil phase in a bioreactor with a magnetic stirrer. We used various stirring rates (100–400 rpm) to assemble individual building blocks of PEG microgels, resulting in forming four types of microgel assembly (i.e., linear, branched, and random assembly with square-shaped microgels and closely packed assembly with hexagonal-shaped microgels). Various stirring times (10–120 s) were also used to control different types of hydrogel assembly. After assembling the individual building blocks of PEG microgels, mineral oil was completely removed from a Petri dish. For further stabilization of hydrogel assembly, the assembled hydrogels were exposed to secondary UV light for 1 s.

Cell culture, cell viability, and image analysis

NIH-3T3 mouse fibroblast cells were cultured with Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and 1% penicillin–streptomycin in a humidified incubator (5% CO_2, 37°C). The cell viability was analyzed by using a live/dead assay (Invitrogen, CA) with calcein AM (green, live cell) and ethidium homodimer (red, dead cell). The live/dead assay solution (2 μL calcein AM and 0.5 μL ethidium homodimer in 1 mL phosphate buffered saline) was applied into a Petri dish containing the cell-laden hydrogel assembly and was subsequently incubated for 10 min at 37°C. After incubation for 10 min, the live/dead assay was completely removed before taking phase contrast images. Furthermore, after secondary photocrosslinking, assembled hydrogels could be easily transferred into a microplate without any physical damage. Phase contrast and fluorescent images were obtained by using an inverted microscope (Olympus IX71), and the images were analyzed by using Image J software. All data were obtained from at least three

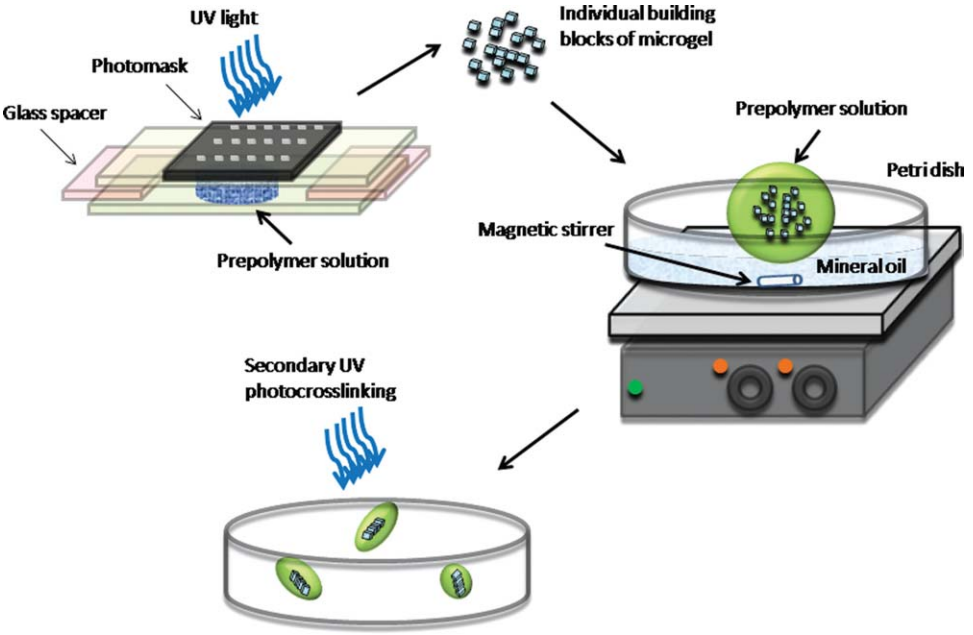

Figure 1. Schematic diagram of hydrogel assembly process.

Individual building blocks of PEG microgels were fabricated by using a photolithography technique. Individual building blocks were subsequently assembled on the two-phase oil-aqueous interface in a bioreactor with a magnetic stirrer. To stabilize the hydrogel assembly, secondary photocrosslinking was performed by using a UV light.

different experiments, and the statistical significance of the data was analyzed by using the Student *t*-test, showing that *P* values (i.e., $P < 0.05$ and $P < 0.01$) were considered statistically significant.

Results and Discussion

To fabricate individual building blocks of PEG microgels, we used the two-phase oil-aqueous bioreactor system (Figure 1). Briefly, individual building blocks of PEG microgels were fabricated by using a UV light through a photomask thin film embedding square and hexagonal shapes (500 μm in length). These individual building blocks of PEG microgels were transferred into a mineral oil phase in a bioreactor with a magnetic stirrer. To enhance the assembly efficiency of hydrophilic PEG microgels, we used hydrophobic and inert mineral oil. We also used OTS-treated glass slides that made the glass surface hydrophobic, showing that individual building blocks of hydrophilic PEG microgels were easily detached from the glass slide. The thickness of these PEG individual building blocks was determined by the height of the glass spacers, indicating that each individual building block showed 140-μm thickness. After primary photocrosslinking process, some prepolymer solution surrounding the individual building blocks of microgels was still remained, and this residual prepolymer solution could facilitate the detachment of microgels from a glass surface. In addition, this residual prepolymer solution was used for secondary crosslinking process without adding any additional prepolymer solution, because the residual hydrophilic prepolymer solution could help to generate self-assembly of hydrogels. After creating hydrogel assembly in a two-phase bioreactor system, the mineral oil was completely removed from a Petri dish. The complete removal of mineral oil before secondary photocrosslinking is useful to avoid the possible oxidation or degradation of mineral oil derived from a UV light. Furthermore, hydrogel-assembled structures can be easily handled by removing mineral oil. Thus, we assembled hydrogels on a mineral oil phase and then completely removed the mineral oil from a Petri dish before the secondary photocrosslinking process.

Various stirring rates and stirring times were used to generate different types of hydrogel assembly. We observed four assembly types of square and hexagonal microgels, such as linear, branched, closely pack, and random. The secondary photocrosslinking process enhanced the stabilization of the hydrogel assembly. The hydrogel assembly was created by minimizing the surface free energy generated between a hydrophilic PEG microgel and hydrophobic mineral oil phase. In this article, we used the hydrophobic effect as a driving force for the hydrogel assembly as previous articles have been described.[9,17] When compared with previous mechanical agitation-based hydrogel assembly system[9] and surface-directed microgel assembly method,[12] our bioreactor system enabled the control of cell-laden hydrogel assembly types in an accurate manner, because the assembly types were precisely regulated by the stirring speed and time. In addition, our two-phase bioreactor system enabled the generation of cell-laden hydrogel self-assembly without any manual agitation or micropatterning technique. Although a previous mechanical agitation method could generate hydrogel deformation,[9] we did not find any mechanical deformation of hydrogel assembly in our two-phase bioreactor system. Furthermore, closely packed hexagonal hydrogel self-assembled structures were created by controlling stirring speed and time. Therefore, this oil-aqueous bioreactor system-based hydrogel assembly containing square and hexagonal shapes could be a potentially useful approach for creating 3D tissue microarchitectures in vitro.

Hydrogel assembly with square shapes

We assembled individual building blocks of PEG microgels by using a two-phase oil-aqueous bioreactor system

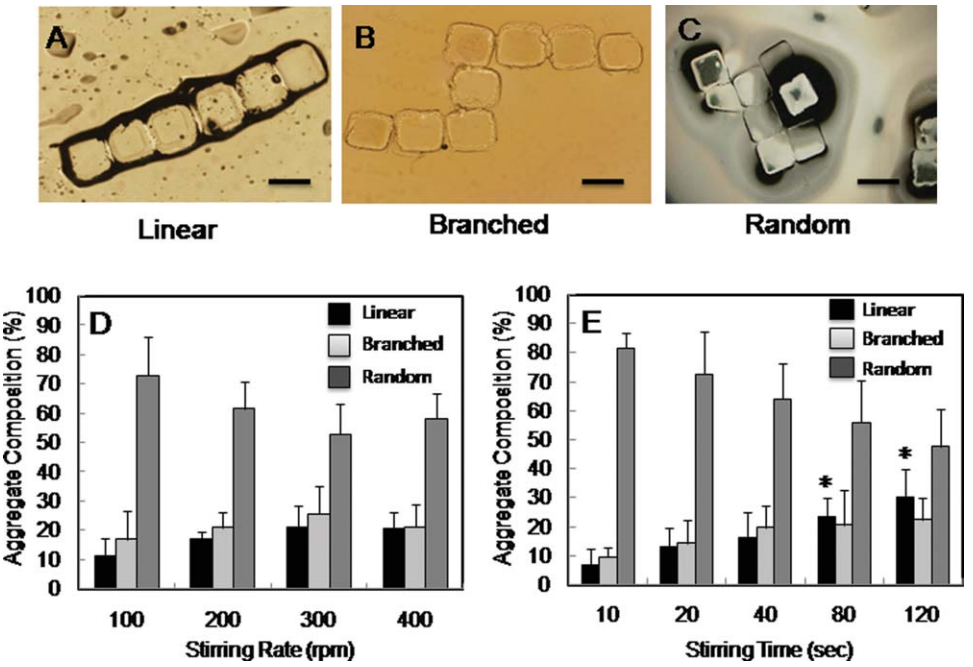

Figure 2. The hydrogel square assembly with three different types.

(A) Linear, (B) Branched, and (C) Random. Scale bars are 500 μm. (D) The effect of stirring rate on hydrogel assembly with square shapes. Eighty seconds stirring time is used. (E) The effect of stirring time on hydrogel assembly with square shapes. 300 rpm stirring rate is used. Data are means $\pm$ standard deviation, $n = 3$, and $^*P < 0.05$.

(Figure 2). Individual building blocks of PEG microgels were directly assembled during the stirring process in a bioreactor, resulting in forming three assembly types (i.e., linear, branched, and random; Figures 2A–C). The types of the hydrogel assembly with square shapes (500 μm $\times$ 500 μm) were controlled by the rotational stirring speed (Figure 2D). We used constant stirring time (80 s) and analyzed the effect of stirring rate on the hydrogel assembly. It was revealed that the aggregation composition percentage of the linear and branched hydrogel assembly was increased with increasing the stirring speed. In contrast, the aggregation composition percentage of random assembly was decreased by increasing the stirring rate. The percentage of aggregate composition means the number of assembled hydrogels (i.e., linear and branched type) divided by the total number of assembled hydrogels including random type. Interestingly, we observed that the aggregate composition percentage of the linear and branched assembly was highest at a 300 rpm stirring rate, whereas random assembly was minimized at a 300 rpm stirring speed. At a 300 rpm stirring rate, the hydrogel assembly percentage with linear and branched types was $\sim$20 and 25%, respectively, whereas random assembly percentage was 55%. The assembly types and aggregate composition percentages obtained from this study were similar to the previously published approach.[9] In the previous approach, the hydrogels were assembled by using a pipette tip-based mechanical agitation on the oil-aqueous solution. However, the types and shapes of hydrogel assembly were not easily controlled, because of the manual agitation of a pipette tip. To overcome this limitation imposed by previous mechanical agitation approach, we used a two-phase oil-aqueous bioreactor system that could regulate the hydrogel assembly in a rapid and controlled manner.

Although we optimized stirring speed (300 rpm) for linear and branched hydrogel assemblies, we did not find higher percentage of hydrogel assemblies. It is probably due to lower hydrophobicity of mineral oil.[18] To enhance the efficiency of the hydrogel assembly, another hydrophobic organic liquid (i.e., perfluorodecaline) that shows higher hydrophobicity and water-repellent property needs to be used. Also, more hydrophilic hydrogels may be beneficial for increasing the percentage of the hydrogel assembly. As expected, the lowest stirring speed (100 rpm) on hydrophobic mineral oil phase enabled the generation of random hydrogel assembly (75%) rather than the linear and branched assembly, indicating 10 and 15%, respectively. When stirring rate was increased (>300 rpm), the percentage of linear and branched assembly remained almost constant. If higher stirring rate (>400 rpm) is used, the linear and branched hydrogel assembly may be broken or more random assembly can be observed. Therefore, we demonstrated that the stirring rate enabled the direct control of hydrogel assembly types, such as linear, branched, and random.

Stirring time also plays a significant role in controlling hydrogel assembly types. After optimizing the conditions of stirring speed (300 rpm) for the linear and branched hydrogel assemblies, we used constant stirring rate (300 rpm) and analyzed the effect of stirring time on hydrogel assembly types (Figure 2E). The quantitative analysis showed that the aggregate percentage of the linear and branched assembly was directly proportional to the stirring time. However, the percentage of the random assembly was inversely proportional to the stirring time. The short stirring (10 s) of individual building blocks of PEG microgels on a mineral oil resulted in lower percentage (10%) of linear and branched hydrogel assembly. However, as stirring time increased, the individual building blocks of PEG microgels generated more linear hydrogel assembly (30%). In case of the random assembly, the assembly percentage was decreased to 50% at a 120 s stirring time. It was revealed that more aligned and organized hydrogel assembly was generated by increasing stirring

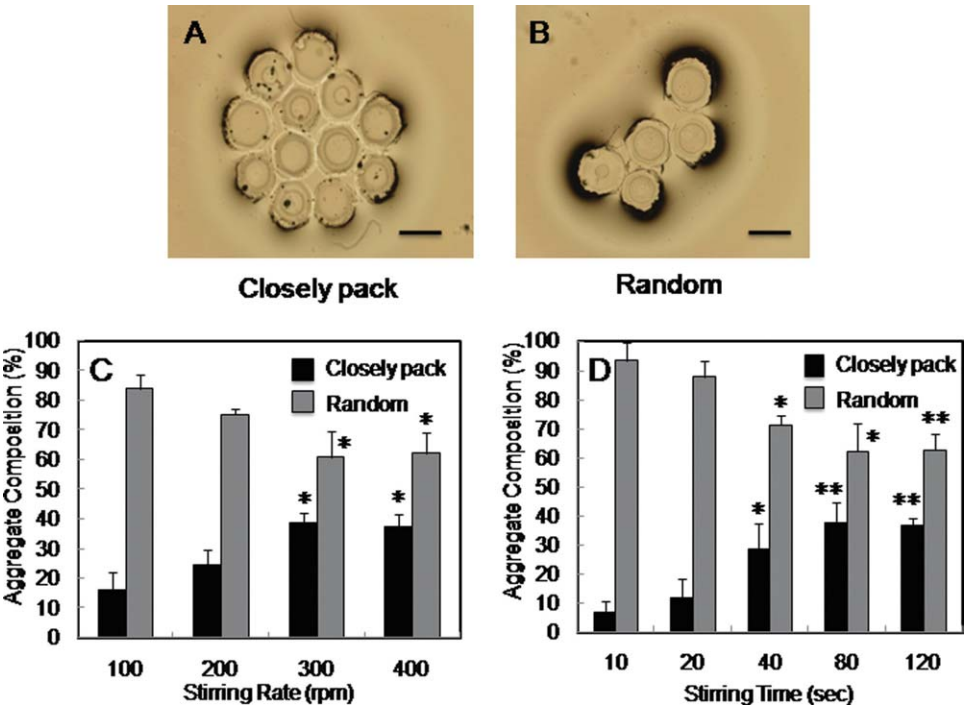

Figure 3. The hydrogel hexagonal assembly with two different types.

(A) Closely pack and (B) Random. Scale bars are 500 μm. (C) The effect of stirring rate on hexagonal hydrogel assembly. Eighty seconds stirring time is used. (D) The effect of stirrering time on hydrogel assembly with hexagonal shapes. 300 rpm stirring rate is used. Data are means $\pm$ standard deviation, $n = 3$, $^*P < 0.05$, and $^{**}P < 0.01$.

time. For the linear assembly, we observed the significant difference of aggregate composition percentage at a 80 and 120 s stirring time when compared with 10 s stirring time ($^*P < 0.05$). Therefore, we demonstrated that the stirring speed and time is an important factor to enable the direct control of the hydrogel square assembly types, such as linear, branched, and random. It was also revealed that the rotational stirring process allowed for the self-aggregation of individual building blocks in a rapid and controlled manner.

Hydrogel assembly with hexagonal shapes

We assembled the individual building blocks of PEG microgels with hexagonal shapes (500 μm in length) (Figure 3). We observed two assembly types, such as closely pack and random (Figures 3A,B). Similar to the hydrogel assembly with square shapes, the stirring rate plays an important role in controlling the hexagonal assembly (Figure 3C). We used constant stirring time (80 s). As expected, at lower stirring speed (100 rpm), the percentage of closely packed hydrogel assembly was low (15%), but we observed high percentage (85%) of random assembly. As stirring rate increased, the percentage of closely packed hexagonal hydrogel assembly was also increased, whereas the percentage of random assembly was decreased. The quantitative analysis showed that the percentage of closely packed hexagonal hydrogel assembly was ~40% at a 300 rpm stirring rate. Thus, we optimized the stirring speed (300 rpm) that could enhance the percentage (40%) of closely packed hydrogel assembly and reduce the percentage (60%) of random assembly. Beyond 300 rpm stirring rate, the aggregate composition percentage of closely packed hydrogel assembly was constant (40%). If we apply at least 300 rpm stirring speed, more high-density closely packed assembly could be generated. We also found significant difference of aggregate composition percentage at a 300 and 400 rpm stirring rate when compared with 100 rpm stirring rate ($^*P < 0.05$).

We analyzed the effect of stirring time on closely packed and random assembly of hexagonal-shaped hydrogels (Figure 3D). We kept the stirring speed (300 rpm) constant. The short stirring (10 s) of individual building blocks of hexagonal microgels provided more random assembly (93%) when compared with closely packed assembly (7%). However, when stirring time increased to 80 s, the percentage of closely packed assembly was significantly increased (35%), whereas the percentage of random assembly was reduced (65%). Beyond 80 s stirring time, the percentage of closely packed and random assembly remained almost constant. We observed significant difference of closely packed aggregate composition percentage at a 80 and 120 s stirring time when compared with 10 s stirring time ($^{**}P < 0.01$). As a result, we confirmed that the assembly of individual building blocks was significantly affected by the surface free energy derived from the two-phase oil-aqueous interface.

From the results of square and hexagonal hydrogel assembly, we optimized the rotational stirring speed (300 rpm) and time (80 s) that could enhance the linear, branched, and closely packed assemblies. Interestingly, we observed that the percentage of closely packed hexagonal assembly was higher (40%) when compared with the linear and branched square assemblies (25%). It is probably due to the square and hexagonal planes, showing that hexagonal and square individual building blocks have six and four planes, respectively. The hexagonal-shaped individual building blocks can easily come into contact with each other because of their larger surface area when compared with square-shaped individual building blocks. Thus, the assembly percentage of hexagonal-shaped individual building blocks was higher when compared with square-shaped hydrogel assembly. We envisioned that the closely packed hydrogel hexagonal

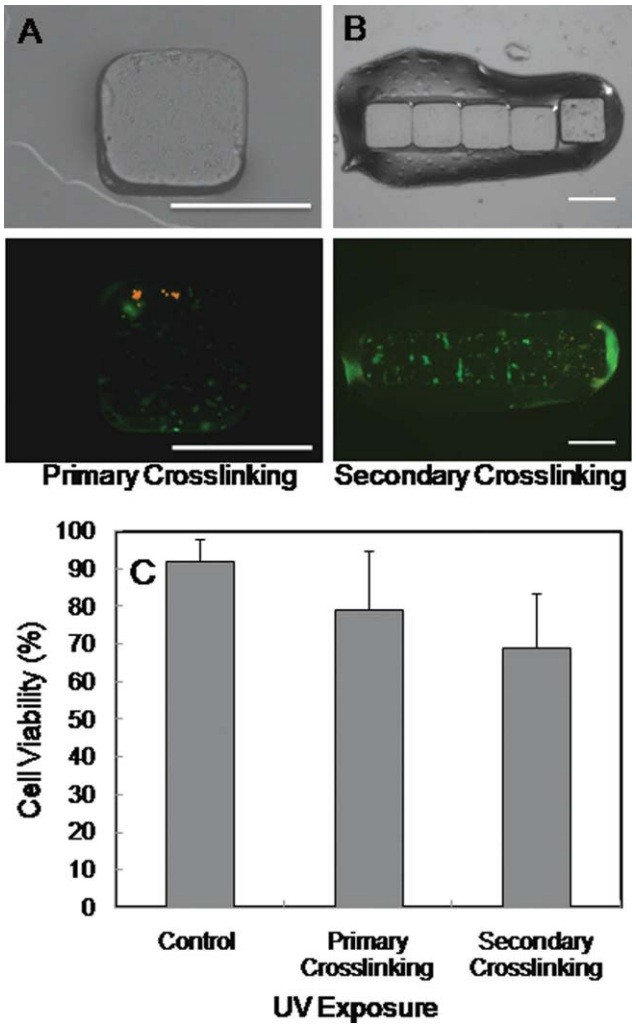

Figure 4. Cell viability of the cell-laden hydrogel assembly.

Phase contrast and fluorescent images after (A) primary and (B) secondary UV crosslinking. Live and dead cells are indicated in green and red, respectively. Scale bars are 500 μm. (C) Cell viability of cell-laden hydrogel assembly containing individual building blocks with square shapes. Data are means $\pm$ standard deviation, $n = 3$.

assembly could be potentially useful for creating 3D liver tissue-like constructs, because the structure of hexagonal assembly is similar to the hexagonal lobules of the liver tissue. Although this bioreactor system-based two-phase oil-aqueous interface approach has the potential to create hydrogel assembly for 3D liver tissue constructs, a number of limitations need to be addressed, such as the inability to generate microvascularized channels within 3D hydrogel-assembled constructs. To address this limitation imposed by a bioreactor-based two-phase oil-aqueous interface approach and to better mimic liver tissue-like functional units, microvascularized channels should be generated within the hexagonal-shaped hydrogel assembly. Therefore, the microvascularized hydrogel assembly with hexagonal shapes could be useful for creating larger liver tissue architectures in vitro.

Cell viability of the cell-laden hydrogel linear assembly

We analyzed the cell viability of cell-laden individual building blocks and hydrogel linear assembly with square shapes using a live/dead assay, such as calcein AM and ethidium homodimer (Figure 4). For the cell-laden hydrogel assembly, 20 μL cell suspension (10 million cells per milliliter) and 20 μL prepolymer solution were mixed and were subsequently photopolymerized by a UV light. We exposed a UV light to 40 μL cell-laden prepolymer solution for 2.5 s. A live/dead assay showed that cells encapsulated within both individual building blocks and hydrogel linear assembly remained viable after primary and secondary photocrosslinking process (Figures 4A,B). The cell viability analysis demonstrated that the viability of the cells encapsulated within the individual building blocks with square shapes was $\sim$80% after primary UV crosslinking (Figure 4C). It was also revealed that although cell-laden microgels were exposed to secondary UV light, we observed higher cell viability (70%) of hydrogel linear assembly with square shapes. Thus, we found that the cell viability after primary and secondary photocrosslinking was relatively high. It is probably due to shorter exposure time of photopolymerization in the primary (2.5 s) and secondary (1 s) crosslinking. We also found that both UV photopolymerization and rotational stirring-based hydrogel assembly processes did not significantly affect the viability of the cell-laden hydrogel assembly. Therefore, this two-phase oil-aqueous bioreactor system-based cell-laden hydrogel assembly could be a potentially useful approach for creating 3D tissue constructs and coculturing different cell types within the self-assembled hydrogel structures.

Conclusions

We developed the two-phase oil-aqueous bioreactor system for generating cell-laden hydrogel assembly. Individual building blocks of hydrophilic PEG microgels fabricated by a photolithography technique were assembled on the hydrophobic mineral oil phase during the stirring process in a bioreactor. We observed three different types (i.e., linear, branched, and random) of the hydrogel assembly with square shapes. The assembly types were significantly regulated by the stirring speed and time. The quantitative analysis showed that the percentage of linear shapes was increased with increasing stirring rate and time. In contrast, the percentage of random assembly was reduced by increasing the stirring rate and time. Similarly, hydrogels with hexagonal shapes were more closely packed with increasing stirring rate and time. Furthermore, cell viability of hydrogel linear assembly with square shapes showed that cells encapsulated within PEG hydrogel assembly remained viable (70%). Therefore, this two-phase bioreactor system-based cell-laden hydrogel assembly could be a potentially powerful approach for creating microscale 3D complex tissue architectures in a rapid and controlled manner.

Acknowledgments

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