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Micromolding of photocrosslinkable chitosan hydrogel for spheroid microarray and co-cultures

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Abstract

Bioengineering approaches, such as co-cultures of multiple cell types, that aim to mimic the physiological microenvironment may be beneficial for optimizing cell function and for engineering tissues *in vitro*. This study describes a novel method for preparing a spheroid microarray on microfabricated hydrogels, alone or in co-cultures. Photocrosslinkable chitosan was synthesized and utilized for fabricating hydrogel microstructures through a micromolding process. The chitosan surface was initially cell repellent but became increasingly cell adhesive over time. By using this unique property of chitosan hydrogels, it was possible to generate patterned co-cultures of spheroids and support cells. In this scheme, cells were initially microarrayed within low shear stress regions of microwells. Human hepatoblastoma cells, Hep G2, seeded in these wells formed spheroids with controlled sizes and shapes and stably secreted albumin during the culture period. The change of cell adhesive properties in the chitosan surface facilitated the adhesion and growth of a second cell type, NIH-3T3 fibroblast, and therefore enabled co-cultures of hepatocyte spheroids and fibroblast monolayers. This co-culture system could be a useful platform for studying heterotypic cell–cell interactions, for drug screening, and for developing implantable bioartificial organs.

Keywords: Spheroid; Chitosan; Hydrogel; Co-culture; Hepatocyte; Fibroblast

1. Introduction

Cells in tissues and organs exist in a three-dimensional (3D) environment surrounded by other cell types. Homotypic and heterotypic cell–cell interactions, as well as individual cellular shapes, play important roles in cellular behaviors such as migration, proliferation, and differentiation. However, most tissue culture techniques lack such morphological and architectural characteristics as cells are typically cultured as single cell types spread out on a two-dimensional (2D) flat surface [1]. The ability to recreate *in vivo*-like microenvironments may lead to advances in diverse fields ranging from tissue engineering to fundamental studies of cell biology [2–4].

Co-cultures of two or more cell types have been used to make more biomimetic environments. These approaches have already demonstrated the importance of heterotypic cell–cell interactions on regulating cell behaviors [5]. Standard co-culture methods that mix two or more cell types, however, cannot be used to easily control the degree of homotypic and heterotypic cell–cell interactions. Micropatterned co-cultures are used to enhance microenvironmental control through spatial localization of multiple cell types relative to each other [6]. In this approach, cells have been patterned on various substrates using photolithography [7,8], microcontact printing [9], inkjet printing [10], and

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microfluidics [11]. Although micropatterned co-cultures have been used to study the effects of cell–cell interactions on various cell functions, one potential disadvantage is that cells are generally patterned on a flat surface and form outspread 2D monolayers. While some cell types such as fibroblasts and endothelial cells actively grow and retain metabolism in 2D monolayer cultures, many cells such as hepatocytes and pancreatic cells frequently lose their organ-specific functions in 2D monolayer cultures and require 3D culture conditions to maintain such functions [12–14]. Thus, co-cultures that are suited for each cell type, such as the combination of 3D and 2D cultures, may be of benefit to enhance efficacy of co-cultures and lead to more advanced tissue engineered constructs.

Spheroid culture, in which cells form 3D multicellular aggregates, has been used to culture cells in 3D environments. For example, hepatocytes forming spheroids have cuboidal cell shapes, reconstruct bile canaliculi, and express intercellular adhesion molecules that are required for cellular communications [15–17]. Hepatocyte spheroids also exhibit liver-specific functions such as albumin secretion, urea synthesis, and drug metabolism for an extended period of time [15,18–20]. Recently, micropatterns of spheroids have been generated using microscale technologies such as micromolding and microfabrication. In these approaches, geometric features such as microwells have typically been fabricated with non-cell adhesive polymers such as poly (ethylene glycol) (PEG). For example, photocrosslinkable PEG hydrogels have been used to form microwells using micromolding techniques [21]. Alternatively, microwells fabricated from other materials can be modified using chemical modification, electrostatic force or physical adsorption with PEG to make these surface cell repellent [17,22]. Such non-adhesive PEG microwells create regions of low shear stress for cell immobilization and subsequent spheroid formation while preventing random cell adhesions on substrate surface [21]. Although these systems allow for 3D cell aggregations of a single cell type, they may not be suitable for co-cultures of additional cell types in a spatially controlled manner because of the non-adhesive property of the polymers.

With the goal of generating spatially controlled 3D co-culture systems, here we used photocrosslinkable chitosan. Chitosan is a hydrophilic and non-toxic polysaccharide [23]. Because of its biocompatibility and similarities to naturally occurring glycosaminoglycans, chitosan is useful for various biomedical applications in tissue engineering [24,25], drug delivery [26], wound healing [27], and surgical adhesives [28]. In this study, we describe micromolding process using photocrosslinkable chitosan and show that the cellular attachment properties are significantly changed from cell repulsive to adhesive. These properties facilitate the formation of spheroids inside the microwells and the subsequent adhesion of a second cell type. This spatially controlled spheroid co-culture system may be useful for fabricating biomimetic cellular microenvironment.

2. Materials and methods

2.1. Materials

Cell lines were purchased from American Type Culture Collection. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Co. Chitosan glutamate (Protasan UP G113; Mw: <200 kDa; degree of deacetylation: 75–90%) was purchased from Novamatrix (Norway). 4-azidobenzoic acid was purchased from TCI America (Portland, OR, USA). All other chemicals were purchased from Sigma, unless otherwise indicated.

2.2. Photocrosslinkable chitosan synthesis

Photocrosslinkable chitosan (Fig. 1) was synthesized using a protocol described previously [29,30]. Briefly, 200 mg of chitosan glutamate, 116.2 mg of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, 70 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 40 mg of 4-azidobenzoic acid were mixed in 20 ml distilled water in a round bottom flask. The reaction was conducted at pH 5 and room temperature overnight. The modified chitosan was dialyzed for purification, then lyophilized and stored at $-20\,^{\circ}C$ until use.

2.3. PDMS molds

The silicon master for preparing the poly(dimethylsiloxane) (PDMS) replica was fabricated with SU-8 photoresist (MicroChem Co.) using photolithography. A PDMS replica was molded by casting the liquid prepolymers composed of a mixture of 10:1 silicon elastomer and the curing agent (Sylgard 184, Essex Chemical). The mixture was cured at $70\,^{\circ}C$ for 2 h, and the PDMS mold was then peeled from the silicon wafer, cleaned with ethanol or acetone, and plasma cleaned for 4 min to increase its wettability (PDC-001, Harrick Scientific Co.).

2.4. Chitosan microstructures

Fig. 2 shows the scheme for fabrication of chitosan microstructures using micromolding. To fabricate hydrogel microstructures with the PDMS mold, a few drops of the macromonomer solution (20 mg/ml photocrosslinkable chitosan in saline) was placed onto a cover glass (Iwaki glass Co., Japan). The PDMS mold was then brought into contact with the solution and gently pressed. Finally, the solution was slowly polymerized into a gel with exposure to longwave UV irradiation (Black-Ray, UVP Inc., radiation range 315–400 nm, peak at 365 nm) for 5 min. After exposure, the PDMS mold was peeled from the surface and the hydrogel was immediately placed in phosphate buffer solution (PBS).

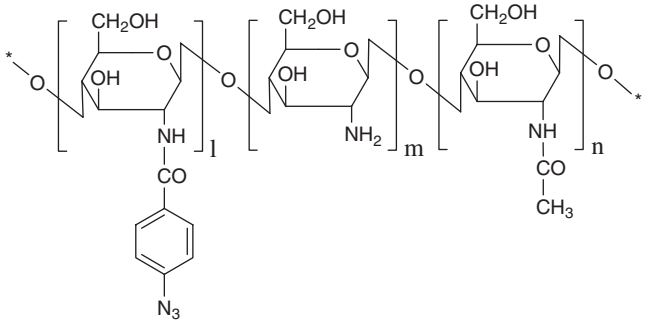

Fig. 1. Chemical structure of photocrosslinkable chitosan.

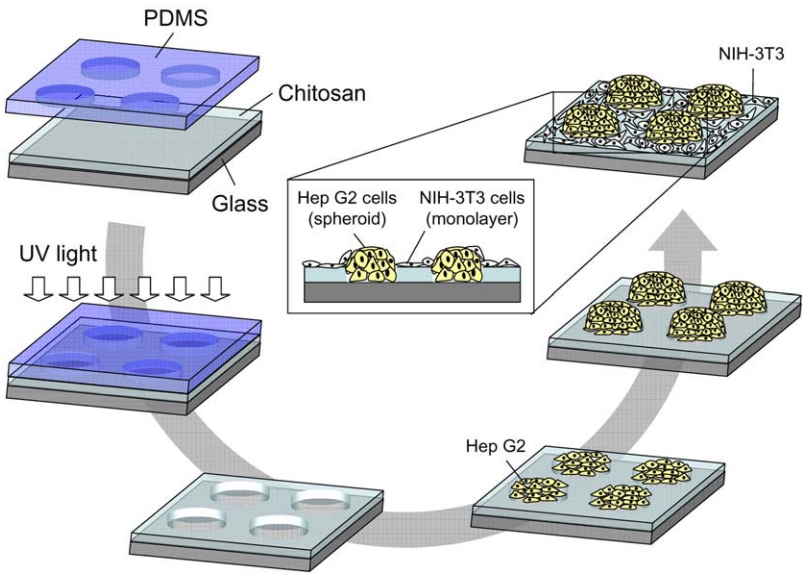

Fig. 2. The schematic of fabrication of spheroid co-culture system using micromolding technology with photocrosslinkable chitosan hydrogel. The chitosan solution was placed on a cover glass, molded with PDMS and crosslinked with UV light. Hep G2 cells were seeded onto the patterned chitosan, and the cells outside microstructures were removed by changing medium. Hep G2 cells were allowed to form aggregates for 24 h, and then NIH-3T3 fibroblasts were seeded and left to attach onto the surrounding chitosan surface. The NIH-3T3 cells attached evenly to the chitosan surface surrounding the Hep G2 spheroids and proliferated over time to cover the surface of the entire hydrogel.

2.5. Cell preparation

Human hepatoblastoma cells, Hep G2, were maintained in DMEM containing 10% FBS and were passaged every 7 days. NIH-3T3 mouse fibroblasts were maintained in the same medium and were passaged every 3 days. All cells were cultured at 37 °C, 5% CO_2 in humidified incubator.

2.6. Cell pre-staining

Hep G2 and NIH-3T3 cells were distinguished by staining with carboxyfluorescein diacetate succinimidyl ester (CFSE) dye (green) and Vybrant Dil dye (red) (Invitrogen Co.), respectively. Cells were trypsinized and washed with DMEM medium without serum, and incubated in 10 μg/mL CFSE in PBS at a concentration of 1×10^7 cells/mL, or in Vybrant Dil solution diluted 1:200 with PBS at a concentration of 1×10^7 cells/mL for 10 min at room temperature.

2.7. Cell attachment on chitosan flat surface

Flat surface chitosan were polymerized on a cover glass with flat surface PDMS. Cover glass coated with 0.3% collagen (BD Bioscience) for 30 min was used as a control. Both the collagen-coated cover glass and the flat surface chitosan were sterilized under UV light exposure in a laminar flow hood and placed in the medium in 6-well plates. Cells were trypsinized, stained with CFSE and seeded at a density of 5×10^5 cells/well. At 0.25, 0.5, 1, 2.5, 6 and 24 h of incubation, cover glasses were gently washed in PBS and transferred into 4% formaldehyde. Fluorescent images were obtained with a fluorescent microscope (Zeiss Axiovert 200), and cells were quantified by counting attached cells in a minimum of three individual experiments at each time point. Cell attachment rates were calculated by dividing the difference of the attached cell numbers between each time period by the time that elapsed.

2.8. Spheroid culture and co-culture

Chitosan microwells (200 μm in diameter and 50 μm deep) were used for the spheroid culture of Hep G2 and the co-culture with NIH-3T3 cells. The chitosan microwells were placed in 6-well plates. Hep G2 cells were trypsinized, stained with CFSE and plated at a density of 2×10^6 cells/well. After 15 min, the chitosan microstructures with cells were gently washed with PBS and transferred to fresh 6-well plates. 24 h after transfer, NIH-3T3 cells were trypsinized, stained with Vybrant Dil and plated for co-culture at a density of 1×10^6 cells/well. As a control, cells were co-cultured in a cover glass coated with 0.3% collagen with the same seeding protocol.

The medium was changed and collected from each sample at days 3, 5, 7, and 9 after the initial Hep G2 seeding for quantification of albumin secretion. The albumin concentration secreted during 48 h in each culture medium was measured by using enzyme-linked immunosorbent assay with commercialized kits (Alb well II, Exocell Inco.). Samples were visualized and imaged on day 9 after initial seeding with a fluorescent microscope (Axiovert 200, Zeiss). Samples were fixed with 4% formaldehyde and transferred to 100% EtOH through a 50%, 60%, 80%, 90%, 100% gradient, and then transferred to 50% and 100% hexamethyldisilizane. The slides were left in 100% hexamethyldisilizane to dry in a chemical fume hood over night. Each sample was sputter-coated with gold and imaged using a scanning electron microscope (SEM, JEOL JSM 6060).

2.9. Live/dead evaluation

A live/dead assay was performed with calcein-AM and ethidium homodimer (Molecular Probes). The two components were added to PBS, each at a concentration of 1 μg/ml. Cells in the chitosan microwells at 9 days of culture were placed in the solution for 20 min. After rinsing with PBS, cells were evaluated using a confocal laser microscope (Bio-Rad, Hertfordshire, UK) with an argon laser light source and double detectors. In the live/dead assay, calcein-AM penetrates into the cytosol of viable cells and stains them green, while ethidium homodimer stains dead cell nucleus red. Viability was calculated from image analysis of 60 slice microphotographs in three independent experiments.

2.10. Data analysis

Data were expressed as means ± standard deviations (SD). Statistical evaluations of numerical variables were performed using unpaired

student's *t*-test for Table 1 and using repeated measures ANOVA for Fig. 6. $P<0.01$ was considered significant.

3. Results and discussion

3.1. Cell attachment on a chitosan hydrogel flat surface

Most synthetic and natural hydrogels such as PEG and dextran are known to prevent cellular adhesion for a long period of time, because most cells do not have receptors to hydrogel polymers [31]. Furthermore, due to the hydrophilic nature of hydrogels, extracellular matrix proteins, such as laminin, fibronectin, and vitronectin, typically do not readily absorb to the hydrogel surfaces. These attributes have been exploited in the application of post-operative adhesion barriers and in the design of specific cell adhesion surfaces [32]. For example, chitosan has attracted attention as a potential post-operative adhesion barrier [33] and has been shown to deter cell attachment and growth *in vitro* [29]. Contrary to these approaches, cell adhesions to a chitosan surface have been reported [34]. There are several efforts to apply chitosan hydrogels for adhesive scaffolds [35,36].

In this study, we initially characterized the attachment properties of a chitosan hydrogel. The rate of cell attachment onto a flat chitosan surface was determined for hepatocytes and fibroblasts. Fig. 3 shows the number of attached cells of the two cell types at different time points. Cell attachment rates are calculated from the difference of the numbers, and the averages in 0–1 h and in 2.5–24 h are shown in Table 1. For both cell types, the chitosan surface was initially cell repellent, as a limited number of cells attached to the flat chitosan surface within the first hour of incubation, while cells attached more readily to the collagen-coated surface. Over time, the chitosan surface became increasingly cell adherent as compared to the collagen surface. The cell attachment to chitosan began to increase after two and half hours of incubation and eventually exceeded that of the collagen surface 1 day after the initial cell seeding. The reason for this change in cell adhesion after a few hours of culture remains unclear, but it is possible that proteins included in FBS and/or secreted by the cells adsorbed to the positively charged chitosan hydrogel. The time-dependent surface cell attachment appears to be a unique property of chitosan, not characteristic of hydrogels such as PEG or dextran.

Table 1
Cell attachment rate calculated from Fig. 3

		Cell attachment rate (cells/h)	
		0–1 h	2.5–24 h
Hep G2	Chitosan	470 ± 374*	$1352\pm545^{\dagger}$
	Collagen	$15\,726\pm5284$*	$65\pm269^{\dagger}$
NIH-3T3	Chitosan	148 ± 39**	$1480\pm131^{\ddagger}$
	Collagen	4033 ± 1491**	$273\pm178^{\ddagger}$

*, **, †, ‡: $P<0.01$.

3.2. Chitosan microstructure and cell patterning

The micromolding process outlined in Fig. 2 used PDMS molds as templates to pattern chitosan polymer. The polymer solution was placed on a cover glass, and the PDMS mold was then brought into contact with the solution and gently pressed. Once the polymers were molded and crosslinked with UV light, the PDMS mold was gently removed. One of the parameters first optimized in the micromolding process of chitosan was the concentration of photocrosslinkable chitosan. Photocrosslinkable chitosan was dissolved in saline in a series of dilutions. At high chitosan concentrations, the polymer solution was too viscous to surround the PDMS mold, and microstructures did not form a well-patterned array. At extremely low chitosan concentrations (<5 mg/ml), the polymer did not crosslink well and instead formed hydrogels that easily

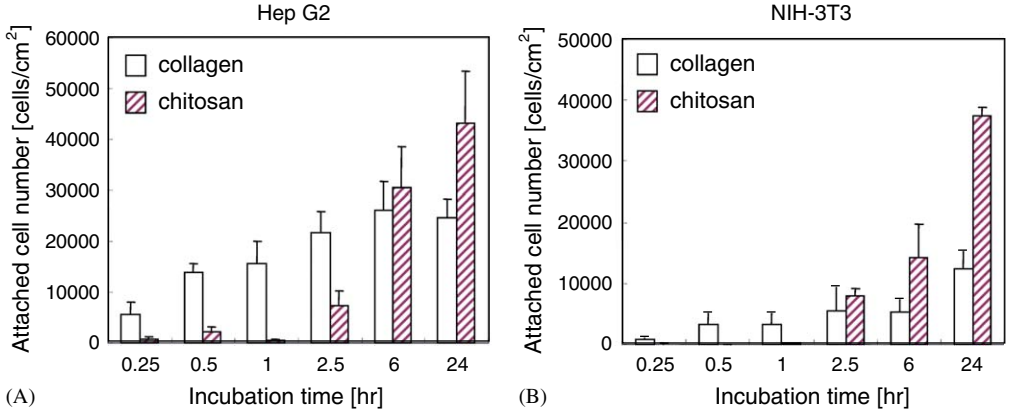

Fig. 3. Cell attachments on a chitosan hydrogel. The attached cell numbers to flat surface of chitosan and collagen were determined for Hep G2 hepatocytes (A) and NIH-3T3 fibroblasts (B). For both cell types, the chitosan surface was initially cell repellent, as a limited number of cells attached to the chitosan flat surface within the first hour of incubation, while cells attached more readily to the collagen-coated surface. Cell attachment to chitosan began to increase after two and a half hours of incubation and eventually exceeded that of the collagen surface one day after the initial cell seeding. The values indicate the mean of a minimum of three individual experiments at each time point. Error bars indicate SD.

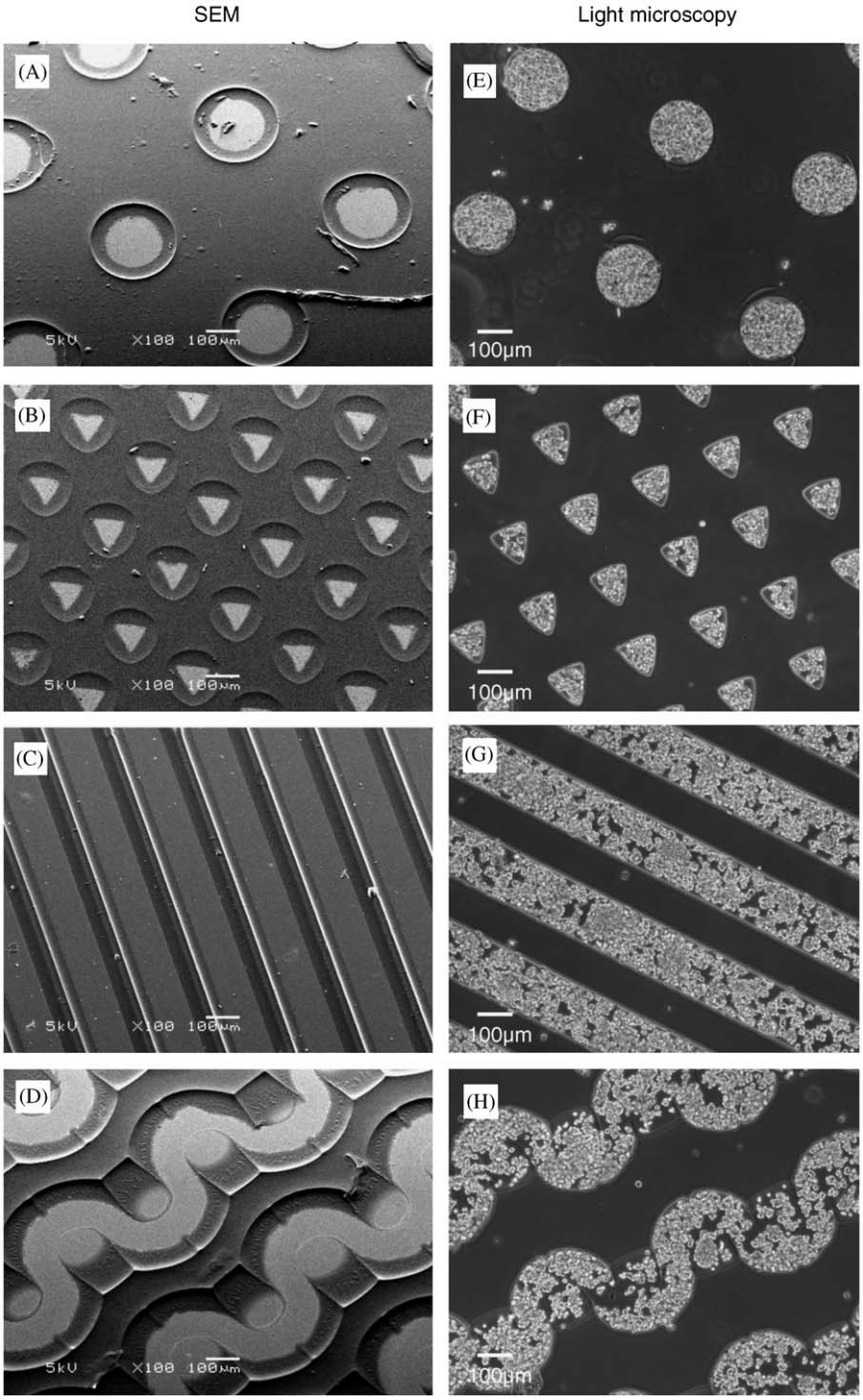

Fig. 4. Chitosan microstructures and cell patterning. SEM pictures show that chitosan microstructures could be made into various shapes such as circle (A), triangle (B), line (C), and curved line (D) using corresponding PDMS molds. Due to the high water content of the hydrogel, structures visualized using SEM were shallower, thinner, and wider than the actual hydrated structures. Light microscope images show that Hep G2 cells were docked within low shear stress regions of these patterned microstructures (E–H).

disintegrated. We found that 20 mg/mL of chitosan was suitable for the micromolding process.

Microfabricated chitosan structures were visualized and characterized using SEM and light microscopy. Chitosan microstructures could be made into various shapes using corresponding PDMS molds as shown in Figs. 4A–D. Hep G2 cells were seeded onto the patterned chitosan hydrogel, and the cells outside microstructures were removed within the first hour of seeding by changing medium so that only cells that had docked in low-shear regions remained (Figs. 4E–H). It is worth noting that structures visualized using SEM were shallower, thinner, and wider than the structures which remained hydrated in culture conditions because samples were dehydrated for SEM analysis. This is

due to the high water content of the hydrogel, which was approximately 98% w/v (20 mg/ml chitosan in saline) in this study. All microarrays shown in Fig. 4 were made with convex PDMS molds; however, it was also possible to make protruding chitosan microstructures with concave PDMS molds (not shown). Developing microfabrication and micromolding techniques for biocompatible and biodegradable polymers using photochemistry can be advantageous for a variety of applications in both tissue engineering and high-throughput diagnostics.

3.3. Hepatocyte spheroid formation on chitosan microstructure

Micropatterned hydrogels provided excellent geometrical structures for 3D cell cultures either alone or in co-culture. Here, we used microarrays of circular wells that were 200 μm in diameter and 50 μm in depth to provide low-shear stress regions in which cells could dock. Hep G2 cells were seeded into the microwells, and the cells outside microstructures were removed within the first hour of seeding by changing medium so that only cells that have docked in low-shear regions remained. One day after seeding, Hep G2 cells began to form aggregates inside the wells (Fig. 5A). By day 3, the aggregates grew into hemispherical spheroids (Fig. 5B), and the spheroids continued to grow in size until their growth was physically constrained by the microwell boundary at the end of experiment (Fig. 5C). Interestingly, cells that settled inside the microwells remained within the wells and did not actively migrate or invade areas outside, despite their affinity towards the chitosan surface as shown in the adhesion experiments in Fig. 3. The reason why cells in the wells preferred aggregation to adhesion onto chitosan may be explained by the work of adhesion. Generally, hierarchy of cell–cell and cell–matrix interactions is described by the work of adhesion between them, which is analogous to molecules in a liquid solution [37]. Cells that express high levels of cell–cell adhesion molecules will have a large work of cell–cell adhesion. In our previous study, hepatocytes forming spheroids strongly expressed intercellular adhesion proteins such as cadherin and claudin during culture [17]. Although the chitosan surface increased in affinity for cellular attachment over time in this study (Table 1), the fact that HepG2s kept the aggregated state means that the work of cell–cell adhesion was larger than that of cell–matrix adhesion. The SEM image shows the round hemispherical portion of the aggregates above the chitosan hydrogel (Fig. 5D).

In our system, phenyl azide groups were introduced to the chitosan polymer (Fig. 1). Upon photolysis, azide groups ($-N_3$) form short-lived nitrene groups that react rapidly with each other or with the amino groups of chitosan to generate azo groups ($-N=N-$). Therefore, this system does not require the use of soluble photoinitiator, which may introduce cytotoxicity and be possible disadvantage in further applications such as cellular encapsulation and implantable artificial organs. Live/dead staining was used to demonstrate the viability of the cells within the aggregates formed on the chitosan hydrogel. $92.6 \pm 4.0\%$ of the cells were viable based on image analysis of 60 slice microphotographs from confocal microscopy. Fig. 5E shows a representative stacked image of the live/dead staining.

Hepatocytes are often phenotypically unstable in culture and lose important metabolic activities [12]. One marker of liver-specific functions is the secretion of albumin. To analyze the phenotypic stability of Hep G2 spheroids, medium from samples was collected on days 3, 5, 7, and 9 for albumin ELISA analysis. While cultured on the

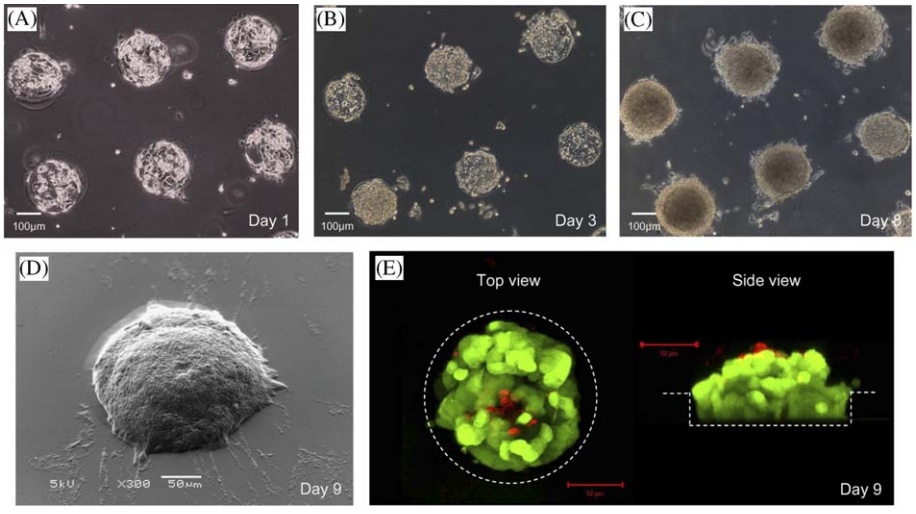

Fig. 5. Hepatocyte spheroid formation on chitosan microstructure. Hep G2 cells seeded into microwells began to form aggregates inside the wells one day after seeding (A). By day 3, the aggregates grew into hemispherical spheroids (B), and the spheroids continued to grow in size until their growth was physically constrained by the microwell boundary at the end of experiment (C). The SEM image shows the round hemispherical portion of the aggregates above the chitosan hydrogel (D). Live (green)/dead (red) viability within spheroid were evaluated by a confocal laser microscope, which shows that most of the cells were alive (E).

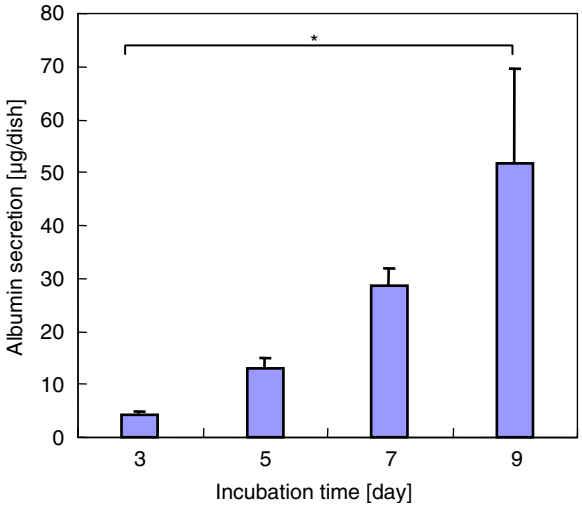

Fig. 6. Albumin secretion of spheroids on chitosan microstructure. The concentration of albumin secreted during 48 h in each culture medium was measured using enzyme-linked immunosorbent assay. While cultured on the chitosan hydrogel, Hep G2 spheroids continuously secreted increasing amounts of albumin over the 9 days of growth. The values indicate the mean of a minimum of three individual experiments at each time point. Error bars indicate SD. $*P<0.01$.

chitosan hydrogel, Hep G2 spheroids continuously secreted increasing amounts of albumin over the 9 days of growth as shown in Fig. 6. These results suggest that chitosan-based microstructures are suitable as a template for spheroid formation and maintenance.

3.4. Co-culture of hepatocyte spheroids and fibroblast monolayer

Based on the time-dependence of cell adhesion to chitosan, co-cultures of Hep G2 and NIH-3T3 fibroblasts were constructed where the two cell types were spatially separated. Hep G2 cells were seeded and allowed to form aggregates for 24 h, after which NIH-3T3 cells were seeded and left to attach to the surrounding chitosan surface. The NIH-3T3 fibroblasts attached evenly to the chitosan surface surrounding the Hep G2 spheroids and proliferated over time to cover the surface of the entire hydrogel (Figs. 7A and B). The SEM image shows that the Hep G2 spheroid maintained its round shape in the presence of the NIH-3T3 fibroblasts (Fig. 7C) in a similar fashion to the Hep G2 single culture (Fig. 5D). Again, the dimensions of the spheroid shown are likely to be smaller than those of the aggregates in hydrated culture conditions. Here, due to the dehydration of the NIH-3T3 and Hep G2 cells, as well as the chitosan hydrogel during sample preparation, the Hep G2 aggregate detached from the neighboring NIH-3T3 monolayer. To distinguish Hep G2 and NIH-3T3 cells in the co-culture system, the two cell types were stained with two different fluorescent cell tags and visualized using fluorescent microscopy (green, Hep G2; red, NIH-3T3). Hep G2 remained within the wells (Fig. 7D), and NIH-3T3 grew adjacent to and over the Hep G2 spheroids on chitosan hydrogels (Fig. 7E), showing homotypic and heterotypic interactions that were spatially controlled in contrast to the random co-culture on collagen coated glass with the same seeding protocol (Fig. 7F). During initial seeding of NIH3T3, some cells may have attached to HepG2 spheroids. In addition, by day 9, some NIH3T3 growing on the chitosan surface have began to invade the edges of the HepG2 spheroids due to the rapid division of the NIH3T3 cells. Judging from the green fluorescence in the live/dead stain, cells in both the NIH-3T3 monolayer and the Hep G2 aggregates were viable through 9 days of culture (Fig. 7G). However, cells deeply embedded inside the spheroids were difficult to image because the range of observation depth of this method is less than 100 μm from the spheroid surface due to the transmission limit of laser [38].

The importance of heterotypic cell–cell interactions on cellular behaviors has already been demonstrated in 2D patterned co-cultures [5]. Increasing heterotypic interactions have been correlated with a relative increase of liver-specific functions such as albumin secretion and urea synthesis [39]. It has also been shown that hepatocytes forming 3D spheroids improve not only their functions but also expression of intercellular adhesion molecules and responsiveness to hormones [17,40]. Thus, it is expected that 3D spheroids grown in the co-culture system may respond with great sensitivity to changes in microenvironments including soluble signals from other cell types and direct intercellular interactions. We have not analyzed cell–cell signal transductions or biochemical responses in our co-culture system.

We focused on the hepatocyte spheroid and its co-culture with fibroblast in this study, but the system may be extended to various other combinations of cell types for investigating cell behaviors and the nature of tissues and organs *in vitro*. For example, stellate cells, another cell type in the liver, invade hepatocyte clusters and make gaps between hepatocytes for vascularization *in vivo* [41]. In the pancreas, endocrine cells construct aggregates of a few hundred micrometers in diameter, called Langerhans islets, and interact with the surrounding exocrine portion. It is also crucial to create suitable environments and conditions *in vitro* for the differentiation of spherical aggregates of embryonic stem cells and neuronal stem cells, known as embryoid bodies and neurospheres, respectively [42]. We believe that the present co-culture system may open new opportunities for such studies in tissue engineering, tissue development and differentiation in well-controlled *in vitro* microenvironments. Our next step is to analyze detailed biochemical responses to the defined co-culture conditions as well as potential mechanisms underlying these responses, clarify roles of other cell types, and further apply our system to create tissue engineered constructs.

4. Conclusion

We demonstrated a novel approach to prepare spheroid microarrays and co-cultures using micromolding technology

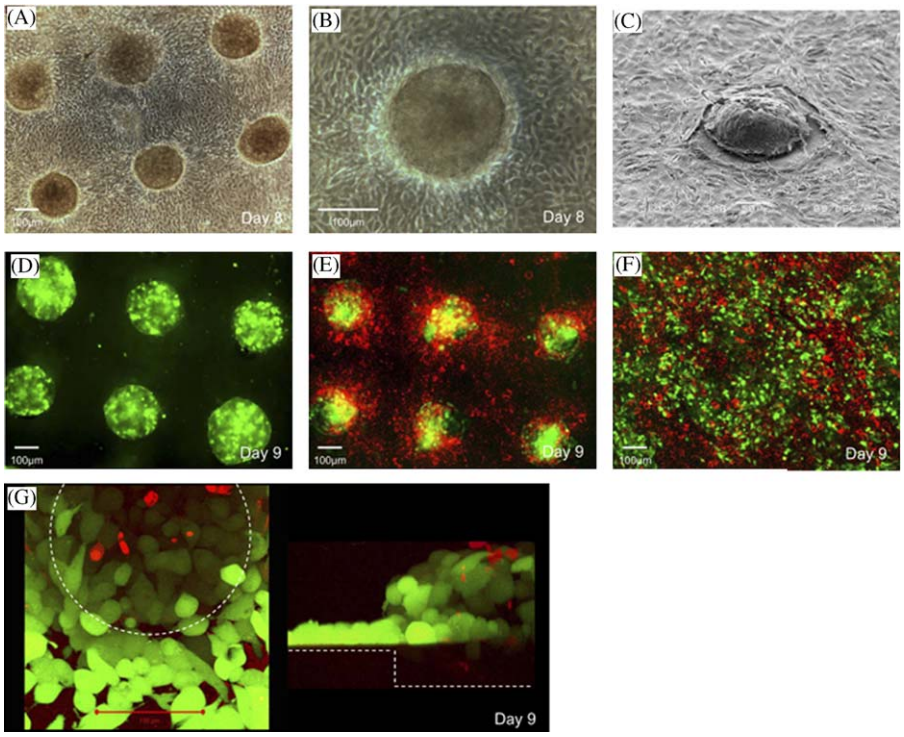

Fig. 7. Co-culture of hepatocyte spheroids and fibroblast cells. Hep G2 cells were seeded and allowed to form aggregates for 24 h, after which NIH-3T3 cells were seeded and left to attach to the chitosan surface. The NIH-3T3 fibroblasts attached evenly to the chitosan surface surrounding the Hep G2 spheroids and proliferated over time to cover the surface of the entire hydrogel (A and B). The SEM image shows that the Hep G2 spheroid maintained its round shape in the presence of the NIH-3T3 fibroblasts (C). Hep G2 and NIH-3T3 cells were distinguished from one another by staining them with two different fluorescent cell tags (green, Hep G2; red, NIH-3T3; yellow, over lap of green and red). Hep G2 remained within the wells (D), and NIH-3T3 grew adjacent to and over the Hep G2 spheroids on chitosan hydrogels (E), showing homotypic and heterotypic interactions that were spatially controlled compared to the random co-culture on collagen coated glass with the same seeding protocol (F). Judging from the green fluorescence in the live/dead stain, cells in both the NIH-3T3 monolayer and the Hep G2 aggregates were viable through 9 days of culture (G), although cells deeply embedded inside the spheroids were difficult to image.

with a photocrosslinkable chitosan hydrogel. The synthesized photocrosslinkable chitosan was compatible with the micromolding processes. Chitosan surface changed significantly from cell-repulsive to cell-adhesive, which facilitated the formation of spheroids inside the microwells and the subsequent adhesion of a second cell type. This spatially controlled spheroid co-culture system could potentially provide a useful tool for fabricating biomimetic cellular microenvironments, for studies of cell–cell interactions, and for tissue engineering applications.

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