장내 미세 환경 모사를 위한 PDMS 채널 내 수화겔 구조의 조립

Assembly of Hydrogel Structure in a PDMS Channel for Intestinal Microenvironment

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for Intestinal Microenvironment
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A thesis submitted to the faculty of KAIST in partial fulfillment of the requirements for the degree of Master of Science and Engineering in the Department of Bio and Brain Engineering. The study was conducted in accordance with Code of Research Ethics.

Daejeon, Korea
2012. 12. 10
Approved by

Professor Je-Kyun Park

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장내 미세 환경 모사를 위한 PDMS 채널 내 수화질 구조의 조립

조 창 현

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ABSTRACT

*In vitro* construction of biomimetic intestinal microenvironment has received much attention due to their special structures and related functions of epithelium. For example, closely packed array of villus and crypt structure increase the total surface area of intestinal epithelium for effective contact with nutrients. The projected structure is crucial for regulating intestinal stem cell proliferation, migration, and intestinal renewal. This structure also provides more effective cell-to-cell interaction within epithelium. In the aspect of drug permeability, gastrointestinal (GI) tract is a primary barrier of first-pass metabolism of oral administered drug. However, microfabrication of long villus structure with high aspect ratio needs complicated fabrication processes using special equipment. In addition, precise control of microenvironment has been limited in conventional methods. In this thesis, we report a novel method of hybrid biomicrofabrication using hydrogel villi structures integrated with microfluidic device to develop a biomimetic intestinal microenvironment system for more physiologically relevant study. A microfluidic device, which was fabricated with poly(dimethylsiloxane) (PDMS), was used in order to develop three-dimensional (3D) cell culture environment under fluidic condition with precise control of reagents and chemicals. We fabricated various sizes of hydrogel villi structures using mixture of alginate and collagen by replica molding from the PDMS mold without any complicated fabrication process. We proposed a latch and hook structure to stably lock-up hydrogel villi structure inside the chamber of a microfluidic device. Assembled hydrogel villi structures were maintained stably under flow condition and showed flexible responses to flow. Human epithelial colorectal adenocarcinoma cell (Caco-2) was cultured on hydrogel villi structures for 7 days and observed that Caco-2 cell adhered to hydrogel surface and proliferated. This hybrid fabrication method may be integrated with other 3D cell culture platform such as organ-on-a-chip, and could be applicable to *in vitro* assay with targeted organ.
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Chapter 1. Introduction

1.1 Biomimetic researches using a chip technology

In recent decades, *in vitro* cell culture issues have been dramatically changed from two-dimensional (2D) monolayer culture to three-dimensional (3D) network in biology and bioengineering field [1]. Most of conventional cell studies had been performed in the form of 2D cell culture. However, 2D cell culture systems have difficulties and limitations in providing in vivo-like environments for the cells, and 2D cultured cells cannot accurately represent a real structure, functions, or physiologies of cells in vivo. Thus, experimental results from 2D cultured cells might be different from *in vivo* test or clinical data. On the other hand, the cells grown in 3D environment have been shown more *in vivo*-like morphologies and physiological functions. Many researchers observed different cellular responses between 2D- and 3D cultured cells that represent the importance of 3D cell culture environment which is more *in vivo*-like environment then 2D cell culture. Pickl et al. reported an effect of 3D cultured cells compared with 2D monolayer cultured cancer cells which overexpress human epidermal growth factor receptor 2 (HER2). They found that trastuzumab (Herceptin), a monoclonal antibody specifically target HER2 showed enhanced inhibition of proliferation towards 3D cultured cancer cells compared with 2D monolayer cultured cells [2]. Sun et al. reported that human skin cells cultured in 3D condition showed a remarkable increase in cell viability against to the oxidative stress and toxicity of heavy metals compared with the same cells grown in two dimensions [3]. These findings emphasized the necessity of 3D cultured cell-based studies in the aspect of providing more
biologically relevant models for cell studies.

In this light, microfluidic systems have become a strong candidate to develop a 3D cell culture system. The capacities for precise control of small volumes of fluids in a microfluidic channel have many benefits over the conventional cell culture systems such as effective delivery of reagents and reactants, continuous and controlled perfusion of nutrients or chemicals, and site-targeted delivery of reagents [4]. More importantly, microfluidic system can provide ideal conditions for the construction of 3D cell culture environment to different kinds of cells by tailored microchannel designing. In this reason, many studies have been reported about the construction of 3D cell culture environment with microfluidics. Choi et al. reported about the microfluidic scaffold with cell-seedable biomaterial for patterned 3D cell culture [5]. This microfluidic scaffold enables convective transport via embedded special microfluidic channel. A stable chemical gradient can also be generated and maintained using a microfluidic network which is designed for the generation of concentration gradient. Microfluidic devices which can culture cells in a long period and manipulate cells using microfabricated structures were reported to mimic *in vivo* cell culture environments, including tissues and organs [6–8]. Other research groups also reported a real tissue-like 3D cell culture system with microfluidic channels, mimicking blood vessel [9], liver [10], lung [11], and kidney [12].

*In vitro* construction of intestinal microenvironment is one of the important issues in biomimetic research field due to its characteristics related to the structures and functions of intestinal epithelium. In the real intestinal environment in human body, closely packed villus
and crypt structures increase the absorptive area which leads to efficient digestion and nutrient uptake. These structures play key roles in regulating intestinal stem cell proliferation, migration, and intestinal epithelial renewal [13,14]. In the aspect of drug permeability test, the epithelial cell monolayer lining the gastrointestinal (GI) tract is considered as a primary barrier for oral administered drug permeation [15,16]. Many drug candidates in the later phase of clinical trials failed due to the different conditions of 2D cultured cells compared to in vivo system [17]. Consequently, research about structural and functional mimetic of intestinal microenvironment is necessary to understand drug deliveries in the human body.

Recently, many approaches have been performed to develop relevant intestinal microenvironment in vitro. The research about the influence of crypt structure on intestinal epithelial cell phenotype was reported [18]. On the PDMS mold which have various size of crypt well, morphology, proliferation, and metabolic activity were measured and compared to 2D flat cell culture. It showed significant difference between the cells cultured on crypt and the 2D flat plate. The metabolic activity of cells was quite enhanced, while proliferation rate of cells decreased depending on the crypt size. Other group reported a 3D microscale GI tract model using collagen scaffold [19]. Collagen cell scaffold with high aspect ratio was fabricated by replica molding using sacrificial layers. Caco-2 cells were seeded on the 3D collagen structures and cultured to form biomimetic intestinal villi which are fully covered with epithelial cells. This research group also performed a drug permeability test using the fabricated intestinal villi model [20]. This result showed that the permeability of epithelial cells in 3D collagen scaffold was much higher than that of the cells cultured with 2D
collagen and conventional method. Fabrication of biomimetic intestinal villus array using poly(lactic acid) (PLA) was reported as well [21]. They cultured Caco-2 cells on the villus array and observed the Caco-2 cell population, viability, and the formation of tight junctions between cells. Viney et al. showed co-culture studies of Caco-2 cell and stromal cells in the 3D hydrogel [22]. They found out that the morphologies of cells that co-cultured in hydrogel showed a significant difference with mono-cultured cells.

Many researchers have developed technologies for the construction of in-vivo like intestinal microenvironment but their approaches have several limitations. First, the fabrication of 3D villus structure with high aspect ratio is difficult due to the complex fabrication process. Typically, laser ablation [19] and UV-lithography, electroplating, and molding (UV-LIGA) [21] process were used in previous works which are costly process and required special equipment. Second, the modeling conditions of intestinal microenvironment were mostly static rather than dynamic. Animal in vivo model and our real GI tract are always active with regular/irregular peristalsis and transported food [23]. It is hard to mimic and control dynamic variables including fluid flow, pressure, or chemical concentration using the conventional platforms. Finally, co-culture studies on physiologically relevant 3D villi structure are insufficient. In order to model more reliable biomimetic intestinal microenvironment, these problems must be solved.
Table 1. Reviews of biomimetic intestinal researches.

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1.2 Research Objectives

In this thesis, we propose an assembly of hydrogel structure in a PDMS channel for the construction of biomimetic intestinal microenvironment using microfluidics technology. To construct a biomimetic 3D cell culture scaffold, we fabricate an intestinal villi structure by simple replica molding process using PDMS mold and alginate–collagen mixture. Villi structure can be patterned in horizontal axis, different from conventional villi structure fabrications that were fabricated in vertical axis. The fabricated hydrogel villi structure stands vertically on a microfluidic device by structural supports. Using this technique, we are able to construct a microfluidic 3D cell culture platform which is integrated with hydrogel scaffold. Villi structure with high aspect ratio can be fabricated without any complicated and specialized fabrication processes such as laser ablation or LIGA process. Facile control of the dimension of villi structure can provide various scaffold sizes in one fabrication process.
Furthermore, our technique can provide high-aspect ratio hydrogel structures which cannot be provided by conventional techniques. Integration with microfluidic system, precise control of fluid with reduced process time can be achieved with our system including controlled transport of reagents and reactants. Moreover, dynamic control of fluid flow inside device induces changes of shear stress that we can monitor shear-induced response of cultured cells. This dynamic culture system offers differentiated advantages such as a construction of more \textit{in vivo}-like environment, facile integration with 3D cell culture systems compared with the conventional 3D cell culture methods which were specialized for static culture.

1.3 Thesis Outline

The thesis consists of four chapters. In Chapter 1, backgrounds and research objectives are mentioned. Chapter 2 describes the device design, fabrication process, sample preparation and experimental setups. Chapter 3 deals with results and discussion. The last chapter is conclusion of this thesis.
Chapter 2. Experimental

2.1 Design and fabrication of microfluidic device

A microfluidic device was comprised of a culture chamber, latch structures, inlet and outlet (Fig. 1). The chamber structure is the part for hydrogel villi integration and cell culture. The length, width and height of chamber were 12150, 3150 and 1000 µm, respectively. To fix hydrogel villi structures stably in the microfluidic channel, latch structures were designed on the side walls of chamber and these structures physically lock up hydrogel villi structures on the microfluidic device. This latch structure consists of long groove where the hook structure of hydrogel villi structure fit, and neck structure as shown in Fig. 1B. The length and width of groove are 3150 and 300 µm, respectively, and those of neck structure are 300 and 150 µm, respectively. The height of latch structure was 130 µm, which is smaller than the height of hydrogel villi in order to grab hydrogel villi structure tightly. The height of chamber is higher than that of latch structures that is hard to be fabricated by conventional soft lithography. Thus we performed a cut-and-bonding process to obtain deep chamber. We fabricated 1 mm thickness device by PDMS replica molding which the height of overall structures are 130 µm (Fig. 1C). Then we followed by plasma bonding of flat PDMS ceiling above it, and cut and removed the ceiling of the chamber to make an open device, followed by plasma bonding of flat PDMS ceiling above it. We could obtain 1 mm deep chamber with latch structure of different height on the same device without complicate fabrication process. Inlet and outlet were used to supply and discharge reagents including media and chemicals. We punched holes laterally with commercially available 0.75 mm diameter puncher and insert 23 G
To fabricate the device, photolithography was used for the PDMS molding shown in Fig. 2A. A negative photoresist (PR) SU-8 2050 (MicroChem Corp, MA) was coated on silicon wafer with 130 µm thickness, and patterned by UV and photomask. The prepared mixture of PDMS and curing agent was poured onto patterned silicon wafer and cured for 1 h at 100 °C on hot plate. After curing, the PDMS was peeled off, cut in each device and punched for inlet and outlet.
Figure 2. Schematic diagram of fabrication process of (a) monolayer fabrication for microfluidic device and (b) two-step fabrication for hydrogel villi structure.
2.2 Design and fabrication of PDMS mold for hydrogel villi

Mold of hydrogel villi structure has four compartments; villi structures, basal part, hook structure and loading well (Fig. 3). The villi structure has various lengths from 300 to 1000 µm which is analogous to real intestinal villi size. The width of villi structures was 150 µm on the bottom of villus and smoothly narrowed to form a curved apical structure. The height of villi structures was 150 µm. The length, width and height of basal part were 4350, 150 and 150 µm, respectively. The hook structure is intermeshed with latch structure of the microfluidic device that physically fixes hydrogel villi inside the chamber of device. These structural supporting structures keep the hydrogel villi structures standing upright and withstand against the flow inside the chamber. The length, width and height of hook structure were 300, 150 and 150 µm, respectively. The loading well structure was used to aid hydrogel mixture loading into the villi structure mold. Since the dimension of the hydrogel structure is narrow, compared with a conventional pipet tip and syringe needle, it can be difficult to load hydrogel mixture without spilling over the mold. The loading well consists of 1 mm diameter well and 1 mm length channel to connect with the hydrogel villi structure.

To fabricate projected hook structure above basal part, we performed two-step photolithography shown in Fig. 2B. First layer of negative photoresist SU-8 was spincoated on the Si wafer with 150 µm thickness and patterned by UV light. First photomask is for the villi structure and basal part. After developing the first layer, second layer of negative photoresist SU-8 was spincoated on the patterned Si wafer with 150 µm thickness and patterned by UV light again. Second photomask is for the hook structures. The PDMS replica
molding process was done to make a microfluidic device.

Figure 3. Design of the PDMS mold for hydrogel villi structure and fabricated hydrogel villi structure. The overall dimension of each structure is shown in (a) top view and (b) side view. (c) The hydrogel villi structure peeled off from the PDMS mold.

2.3 Cell culture and sample preparation

Human epithelial colorectal adenocarcinoma cells (Caco-2) were grown in minimum essential media (MEM, Gibco, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen/Strep, Gibco). Cell cultures were maintained at 37 °C in a 5% CO₂ and 95% air humidified atmosphere. Medium was replaced after 2 days of seeding and every 2 days in culture period. Before cell seeding on the hydrogel villi structure, a sample preparation step was needed to optimize the conditions for cell seeding. Caco-2 cell was cultured for 7 days on conventional 100 mm petri dish to obtain enough number of cells. To detach cells, 1 ml of 0.05% trypsin-EDTA (Gibco) was treated and incubated in cell culture incubator for 2 min. Cells were then detached from dish with 10
ml of media, centrifuged to gather cells in the bottom of the tube, and remove supernatant media and trypsin-EDTA. High concentration of cell was needed for the effective cell adhesion to the hydrogel villi structure; cells were concentrated to 1 to $1.5 \times 10^6$ cells/ml, which is higher concentration than that used in the conventional cell seeding protocol.

2.4 Fabrication of the alginate–collagen hydrogel villi structure

Since PDMS has hydrophobic characteristics on its surface and sticky to some kinds of hydrogel, loading and detachment of hydrogel from the PDMS mold can be a technical problem. To facilitate hydrogel loading and detachment, the surface of PDMS mold was treated with plasma for 1 min, followed by surface coating with 6 wt% pluronic F-127 (Sigma, St. Louis, MO, USA) dissolved in distilled water for 30 min and mildly washed on running distilled water.

The overall schematic processes for the fabrication of villi structure are shown in Fig. 4. Hydrogel villi structure were fabricated by mixture of alginic acid sodium salt from brown algae (Sigma) dissolved in phosphate buffered saline (PBS, Gibco) and collagen type I from rat tail (BD Biosciences, San Jose, CA, USA) with the ratio of 1:1. Hydrogel mixture was injected to the loading well of the mold by using syringe. After the loading, hydrogel mixture in the mold was incubated in cell culture incubator for 1 h for the collagen crosslinking, followed by nebulizing 100 mM CaCl$_2$ solution dissolved in PBS for 1 min for the alginate crosslinking. After polymerization of both hydrogels, loading well structures were removed by cutting and mold was immersed in media to maintain moisture and calcium ion concentration. After the polymerization process, hydrogel villi structures were peeled off.
from the mold by mild pipetting.

Figure 4. Fabrication process of the hydrogel villi structure. (a) The PDMS mold was plasma treated for hydrophilic surface which facilitate hydrogel loading. (b) Hydrogel precursor was loaded to the loading well of PDMS mold using syringe. (c) The PDMS mold with hydrogel precursor was incubated in cell culture incubator, followed by nebulizing crosslinking agent. (d) After crosslinking hydrogel, the loading well structure was cut and removed. (e) The fabricated hydrogel villi structure was peeled off by mild pipetting.

2.5 Integration of hydrogel villi with microfluidic device

Fig. 5 shows the integration process of hydrogel villi structure with the microfluidic device. To prevent bubble formation inside the microfluidic device that can disturb fluid flow inside microfluidic channel and robust cell culture, the device was plasma treated for 30 s and filled with media before integration of the hydrogel structures. The fabricated hydrogel
villi were transported to the chamber of device by using pipet and amputated yellow tip, and aligned to match hook structures and latch structures. This handling process was carried out with 30G syringe needle modified for this handling process. Then the chamber was covered with glass slide not to leak hydrogel villi and media. The assembled device and glass slide were docked on weight system and tightly sealed with weighting block on them shown in Fig. 6.

Figure 5. Schematic diagram of the integration process of hydrogel villi and microfluidic device. Hydrogel villi were transported to the chamber of microfluidic device and aligned to match a hook and latch structure. After alignment, the chamber was covered with glass slide to seal the chamber.
Figure 6. Schematic diagram of a device docking platform. Leak-proof sealing was completed by pressure with a weight.

2.6 Construction of on-chip intestinal microenvironment

We performed two different approaches to integrate Caco-2 cell-seeded hydrogel villi structure with the microfluidic device. The first method is to seed and culture cells on the hydrogel villi which already integrated with the microfluidic device. Prepared cells were loaded to syringe and injected to microfluidic device which is already integrated with hydrogel villi. After the cell injection, the device was incubated in the incubator for 1 h for the cell adhesion to hydrogel villi. To remove cells not adhered on the hydrogel villi, media was exchanged with high flow rate. Cells were maintained at 37 °C in a 5% CO₂ and 95% air humidified atmosphere for 7 days with daily media exchange.

The other method is to seed and culture cells on the hydrogel villi structures outside the device. After enough cell preparation, hydrogel villi covered with cells were then integrated with the device. The fabricated hydrogel villi were transported to 24-well plate with minimal media, and 20 µl of prepared cells were dropped onto hydrogel villi. Well plate was
incubated in cell culture incubator for 1 h for cell adhesion to hydrogel villi, and then 1 ml of media was injected to each well. Cell culture condition was the same as the first method.
Chapter 3. Results and Discussion

3.1 Fabrication of hydrogel villi

One of the important issues in this thesis is to fabricate hydrogel villi structures which have a high aspect ratio without complicated or expensive fabrication processes. By using a replica molding technique of hydrogel mixture on the PDMS molds with laterally patterned villi structure, we can easily fabricate hydrogel villi structures which had long villi size with various aspect ratios. It is hard to observe hydrogel villi under the bright field imaging using a conventional microscope due to its bright color and transparency, dark field imaging mode of stereoscope (SZX2-ILLD; Olympus, Japan) was used to observe hydrogel villi structures. Fig. 7 shows the cross-linked hydrogel villi on the PDMS mold before and after cutting, and freely immersed in media. It showed that the projected 3D villus structures and hook structure were constructed well without notable deformation. Fig. 7A and B represented that the loading well cutting process does not damage to original villi structure. To demonstrate the easy size-controllability of the hydrogel villi that we proposed in this thesis, we fabricated several types of hydrogel villus of different dimensions. *In vivo* intestinal villus have various length range from 300 to 1000 μm [24], we chose four length types of villi structure; 300, 450, 650, and 1000 μm and their aspect ratios were 2, 3, 4.33, and 6.77, respectively. In Fig. 8, we verified that hydrogel villi were successfully fabricated regardless of their aspect ratios. This suggests that conventional fabrication process for structures having high aspect ratio structures would be circumvented via a simple process.

The shrinking phenomenon of hydrogel scaffold was observed just after CaCl₂
nebulizing for alginate crosslinking. It showed 5% to 10% size reduction compared with the hydrogel villi and PDMS mold. After the mold was immersed in media, however, the reduced hydrogel villi returned to their original size again. Few minutes after media supply, hydrogel villi were tightly fitted to mold. It seems that these phenomena resulted from the movement of water across hydrogel villi structure. Hydrogel villi structure was fabricated of alginate–collagen mixture and the gelation of alginate was based on electrostatic crosslinking by calcium ion. The difference of ion concentration between the hydrogel and surrounding media causes an ion concentration gradient which induces the movement of water. This movement of water causes swelling and shrinkage of hydrogel villi structure.

![Figure 7. Fabricated hydrogel villi (a, b) on the PDMS mold and (c, d) freely immersed in media. (a) and (b) show hydrogel villi before and after the loading well cutting process, respectively. We found that the loading well cutting do not damage to original structure of hydrogel villi. (c) and (d) show that projected villus and hook structure were well fabricated. The length of scale bars of (a), (b), and (d) are 1 mm, and that of (c) is 2 mm.](image-url)
Figure 8. Various size of the hydrogel villi structure on the PDMS mold. We verified that hydrogel villi were successfully fabricated regardless of their long dimension and aspect ratio which was limited in conventional methods. The length of scale bars of (a–d) were 1 mm, and that of (e–h) were 500 µm.
3.2 Assembly of hydrogel villi and a microfluidic device

We assembled hydrogel villi structures in a microfluidic chamber and completed the assembly with a glass slide as bottom for the microfluidic platform. One of the key points of this assembly process was a latch structure which hydrogel villi and a device physically locked up to fix hydrogel villi structures inside a device. We verified that hydrogel villi structures were arranged and maintained in a device by intermeshing latch structures shown in Fig. 9. This latch structure enables the hydrogel structure assembly in a microfluidic device platform. Another key point was a docking platform which was exploited to seal the microfluidic chamber on the glass slide with physical pressure provided by weight. In conventional methods to make fluidic channels, a channel-embedded substrate was irreversibly bonded with a bottom substrate, thus it was difficult to integrate 3D scaffolds inside an already-bonded channel. However, by employing a latch structure and a docking platform, we were able to overcome this issue. We verified that no leakage was observed with this docking platform within the various conditions of flow rate, including cell seeding, washing, and media exchange.
Figure 9. Hydrogel villi structures were assembled in a chamber of a device. (a) Hydrogel villi structures were arranged to form villi array based on *in vivo* intestinal villi. (b) Latch structures of hydrogel villi were intermeshed with groove of a device. (c, d) Close-up views of latch structures. Dimensions of the latch of hydrogel villi and the device physically locked up Methylene blue solution was used to aid visualization by blue color.
To verify the feasibility of hydrogel villi structure integration with the microfluidic device, a preliminary experiment was performed to check the stability of integration with small number of hydrogel villi. At first, one hydrogel villi was integrated to show the stability of hydrogel villi structure within the device. Originally, hydrogel structures have bright color with little transparency; we used 80 mM methylene blue solution diluted in distilled water to visualize hydrogel villi structure well. As shown in Fig. 10A, the hydrogel villi structures stood upright with structural support of the intermeshed latch-hook structure, which was similar to in vivo intestinal villi structure. And these structures prevented hydrogel villi from sweeping away by fluid flow inside the chamber. After that, we performed a feasibility test of hydrogel structure integration with three hydrogel villi structures. The interval between hydrogel villi and arrangement was based on in vivo intestinal villi structure. Fig. 10B shows three hydrogel villi stood upright the same as one hydrogel villi test. Moreover, they maintained their arrangement without losing their posture. According to these results, we concluded that more hydrogel villi can be integrated stably and they will maintain their arrangement. This shows the potential of the in vitro construction of in vivo like intestinal villi array structure within the microfluidic platform.
Figure 10. (a) One and (b) three hydrogel villi integrated with the microfluidic device. In both cases, the hydrogel villi stood upright with structural support of a latch-hook structure and stably maintained within device. The length of scale bar is 500 μm.

To verify the relevance of mechanical properties of hydrogel villi structure as a biomimetic unit, we performed elasticity test by monitoring the deformation of hydrogel villi under fluid flow. Three hydrogel villi in the device were used in this test. We selected four conditions of flow rate; 0, 30, 50, and 100 μl/h where the flow velocity of small intestine is 1 m/h, which is the same with 30 μl/h in our microfluidic device [25] Methylene blue solution was selected to be injected via syringe for visualization, and the flow rate was controlled by a syringe pump. As a flow rate in the microfluidic chamber increased, hydrogel villi structure deformed more, so that we indirectly verified the deformation extent with respect to flow rate. As shown in Fig. 11, integrated hydrogel villi structures were deformed as the flow rate increased. We observed that the deformation have a tendency to the applied flow rate. Compared with conventional structural mimetic support using rigid materials which are lack of mechanical similarities with real villi, this elasticity test shows the possibilities to mimic
structural and mechanical properties of real environment. In addition, cell cultured on structurally and mechanically relevant scaffolds may represent more \textit{in vivo}-like shear-induced response. In our result, however, the basal part of hydrogel villi were not fully anchored on the bottom glass slide resulted in hydrogel leaning toward fluid flow shown in Fig. 11. If hydrogel scaffolds can be fixed on the bottom by using surface coating or effective structural locking, more biomimetic environment can be provided with our method.

Figure 11. Deformation of hydrogel villi under flow condition; (a) 0 µl/h (static), (b) 30 µl/h, (c) 50 µl/h, and (d) 100 µl/h. For visualization, methylene blue solution was used to be injected. The length of all scale bars were 500 µm.
3.3 Platform stability under flow condition

After investigating the feasibility of flow-based assay using our hydrogel villi assembly platform, we verified the stability of hydrogel villi structures integrated inside a device under various flow rates. We assembled ten hydrogel villi structures in a device and applied five different flow rate conditions. Four types of hydrogel villi which have different aspect ratios were classified by size and performed the same flow conditions. In Fig. 12, each column meant the flow rate conditions which were 0, 30, 50, 100, and 200 μl/h from left to right, respectively. Each row meant the aspect ratios of hydrogel villi which were 2, 3, 4.3, and 6.7 from top to bottom, respectively. As shown, hydrogel villi structures inside a chamber maintained their structures and positions against flow. Every type of hydrogel villi structures withstand even under high flow rate regardless of their aspect ratios. It emphasized that our assembly technique can serve a stable platform to perform flow-controlled studies. Using this platform, dynamic shear-induced response studies under continuously varying flow rate also achievable which have been difficult in conventional 3D cell culture environment.
Figure 12. Hydrogel villi structures which have four different lengths were applied flow conditions. Each row had different aspect ratio; 2, 3, 4.3, and 6.7 from top to bottom, respectively. And each column had different flow rate; 0, 30, 50, 100, and 200 μl/h from left to right. It showed that our platform can stably maintained under mild and even extreme flow condition regardless the aspect ratio of hydrogel villi. The length of scale bars were 500 μm.

3.4 On-demand construction of heterogeneous condition

One of the outstanding advantages of our technique is the flexibility of assembly that we can easily control the number or types of hydrogel villi integrated with a device. In most conventional intestinal mimetic researches, villi structures were fabricated as a whole villi array form so villi array from the same fabrication process have the same dimension. It means that the constructed environment from conventional methods reveal homogeneous condition, where all cells, tissues, and organs have morphological heterogeneous conditions in vivo environment. To mimic in vivo-like heterogeneous environment, we assembled various hydrogel villi structures of different dimensions in the same device and constructed
heterogeneous hydrogel villi arrangement. Fig. 13A shows that four types of hydrogel villi structures were assembled in order. Fig. 13B shows the side view of heterogeneous assembly. It showed that the lengths hydrogel villi structures varied like a mountain chain. Not only regular arrangement showed in Fig. 13, we can freely assemble based on various numbers of cases with controlling size, shape, and spacing that limitless heterogeneity model could be achieved. This on-demand construction of heterogeneous condition may offer a novel platform for case studies such as disease modeling, controlled culture model.

Figure 13. Heterogeneous assembly of hydrogel villi structures in a device. (a) Hydrogel villi structures with four different lengths were arranged in a chamber of a device. (b) Side view of the heterogeneous assembly. Different lengths of hydrogel villi were arranged like a mountain chain. The length of scale bar was 300 μm.
3.5 Caco-2 cell culture on hydrogel villi

Caco-2 cells seeded on hydrogel villi structure inside the microfluidic device were cultivated for 7 days and monitored daily with microscope. Fig. 14 shows the Caco-2 cell proliferation within chamber of the device after 1 day (Fig. 14A and C) and 7 days (Fig. 14B and D). Many cells were not adhered to the surface of the hydrogel structure in 1 day culture. Caco-2 cell is adherent cell that should adhere to any surface for the growth and proliferation, thus these cells that were not adhered were washed out from the chamber by fluidic media exchange. We observed that cells on the bottom glass slide were proliferated well and showed similar morphology to conventional 2D cultured cells. On the other hand, cells on hydrogel villi structure showed less proliferation rate compared with the cells on the glass. This may due to the difference of surface area where cells adhere to. The total surface area of hydrogel villi structure was quite small compared with that of the bottom glass slide. So cells have much probability to adhere the bottom rather than the hydrogel villi structure. Furthermore, cells suspended in media sank down to bottom by gravity; it is hard for the cells to adhere the side of hydrogel villi structure. These reasons may contribute more cells to adhere on the glass surface, rather than on the hydrogel villi structure.

Caco-2 cell culture on hydrogel villi structure outside the device showed quite different results from the culture inside the device. As shown in Fig. 15, cells adhered to the surface of hydrogel villi structure and proliferated well while maintaining their attachment to hydrogel surface. Due to the small volume of droplet culture, the surface area of droplet was much smaller than that of the chamber of device. This indicate that cells with droplet culture
method have much probability of adhesion on the hydrogel villi structure compared with the cells cultured inside the device. Moreover, hydrogel villi structures with droplet culture method were attached to the bottom of well plate, cells that sank down to the bottom easily adhere to the hydrogel structures.

Figure 14. Caco-2 cell culture on hydrogel villi structure integrated with microfluidic device for (a, c) 1 day and (b, d) 7 days after seeding. Cells were not much proliferated and many cells which were not adhered to surface were observed in 1 day culture. In 7 day culture, however, most of cells were adhered and proliferated well. The length of scale bars of (a) and (b) were 500 μm, and that of (c) and (d) were 250 μm.
Figure 15. Caco-2 cell culture on hydrogel villi structure outside the microfluidic device for 4 days. Cells adhered to the surface of hydrogel and proliferated well in contrast with the cells cultured inside the device. The length of scale bars of (a), (c), and (e) were 500 μm, and of (b), (d), and (f) were 100 μm,
Chapter 4. Conclusions

In this thesis, we introduce a novel method of biomicrofabrication to integrate hydrogel structures with a microfluidic device. In conventional microfabrication method, there are some limitations to construct certain shapes of microstructures that are supposed to mimic specific in vivo environment. Particularly, in vitro construction of an intestinal microenvironment is important due to their structures and functions related to epithelium, including nutrients absorption and transportation, regulation of intestinal stem cell proliferation, and a primary barrier for oral administered drug permeation. However, it is limited to make long dimension of villi structure with ultra-high aspect ratio in conventional method and to apply flow condition to already constructed microenvironment. With this technology, we assembled micropatterned, free-standing hydrogel units and PDMS microchannel and easily construct in vivo-like intestinal microenvironment which have intestinal villi structures with high aspect ratios under flow condition. Hydrogel villi structures were fabricated by simple replica molding form the PDMS mold which villi structures were patterned laterally. Hydrogel mixture was loaded to the mold by the loading well structure and a crosslinking agent was nebulized on it. Loading well structures were cut and removed after crosslinking, fabricated hydrogel villi structures were peeled off. By using replica molding with laterally patterned mold, we successfully fabricated four different dimension types of hydrogel villi structures with ultra-high aspect ratio.

We first demonstrated the integration of hydrogel structures with microfluidic device for facile construction of 3D cell culture environment. Hydrogel villi structures were integrated
onto the microfluidic device, stood upright by structural support, and maintained their position. For the structural support, a hook structure of villi structure and a latch structure of a microfluidic device were proposed. These two structures intermeshed and stably lock up hydrogel villi structures inside a microfluidic device. The feasibility test to verify the mechanical properties of hydrogel villi structures, we observed the deformation of hydrogel villi structures under different flow condition. Hydrogel villi structures showed different deformation extent to various flow conditions and it suggested the potential of our platform to observe the shear-induced response under precisely controlled fluid flow within microfluidic device. In a stability test using our platform, four different dimension types of hydrogel villi structures were stably maintained their structures and arrangement under mild to extreme flow rate regardless of their aspect ratios.

On the basis of this hybrid hydrogel platform, we cultured Caco-2 cells onto hydrogel villi structures inside and outside of microfluidic device to mimic in vivo-like intestinal microenvironment. Cell culture inside the device showed not much adhesion and proliferation of cells on hydrogel villi structure due to its small surface area relative to the bottom glass slide. Nevertheless, we found that the hybrid system integrated with hydrogel structures and a microfluidic device can serve as a new 3D cell culture platform. Furthermore, cell culture on hydrogel villi structure outside of device showed well adhesion and proliferation while attached to the surface of hydrogel. These two approaches of our platform for cell culture on hydrogel villi may offer a selective mode based on researcher’s needs. On-chip culture method may be used for monitoring 3D cell culture studies under controlled
environment, including flow rate, ingredients, drugs and chemicals. Off-chip culture method may be used as an assay kit by assembly of already well-proliferated cell-scaffold or tissue section. And combining these two approaches, a tailored-modeling for more complicated conditions can be achievable.

There are some issues remained to be handled for the construction of more relevant biomimetic microenvironment. The dimension of the microfluidic channel and flow inside the chamber should be reconsidered based on fluid dynamics simulation to construct more *in vivo* like physiologies. In the point of cell culture on hydrogel villi structures inside a microfluidic device, cell adhesion and culture conditions are needed to be optimized since it showed lower adhesion rate inside a device compared with culture outside a device. Nevertheless this hybrid assembly technology has much potential for biomimetic researches and can serves a facile 3D cell culture system that is integrated with microfluidic system. Lateral patterning technique of hydrogel villi structure can offer much more flexibilities of the shape of hydrogel villi structures which have been limited in conventional fabrication processes used in other intestinal researches. By changing the composition of hydrogel mixture, various types of hydrogel villi structures with different physiological and mechanical properties can be fabricated. These diverse controllable factors may offer on-demand modeling of various types, including disease modeling and personalized case studies. The integration ability of our system may be used in not only intestinal researches, but also other targets such as lung and liver. Moreover, our technology can be applicable to further 3D cell studies, including *in vivo*-like *in vitro* drug assay, and organ-on-a-chip researches.
본 연구에서는 수화젤 구조체를 PDMS 채널과 조립하는 방법을 이용하여 장내 미세 환경을 유체 환경 내에서 구현하는 방법을 제안하였다. 기존에는 장내 미세 환경의 구조적인 모사에 집중하여 실제 장내에서와 같은 여러 세포들의 공생배양 밑 유체 환경의 구현에 어려움이 있었으며, 높은 형상비(Aspect ratio)를 가진 긴 융털 구조를 제작하기 위해 특수한 장비들을 사용해야 하는 복잡한 공정 과정을 거쳐야 하는 한계점이 나타났다. 이런 단점을 극복하기 위하여 수평 방향 공정 방법으로 제작한 수화젤 구조물은 PDMS 채널과 결합함으로써 기존의 복잡한 공정과정 없이 다양한 크기의 융털 구조를 유체 환경 내에서 구현하였다. 본 실험에서는 장내 융털을 모사한 수화젤 구조체로 알긴산염(Alginate)와 콜라겐을 1:2 로 섞은 혼합물을 사용하였으며, 서로 다른 네 가지 크기의 수화젤 융털 구조를 제작하여 수화젤 구조의 크기 조절이 용이함을 보여주었다. 제작된 수화젤 융털 구조는 미세유체소자와 결합되어 실제 장내 환경과 같이 유체 환경 내에서 수직 방향으로 고정되어 유지됨을 보였다. 또한 미세유체소자 내의 유체가 흘러감에 따라 수화젤 융털 구조가 유연하게 휘어짐을 관찰하였다. 장내 미세 환경을 모사하기 위해 수화젤 융털 구조 표면에 대장암(Human epithelial colorectal adenocarcinoma, Caco-2) 세포를 배양한 결과 세포들이 성공적으로 부착하여 증식하는 것을 확인하였다. 하지만 수화젤 융털 구조와 결합된 미세유체소자에 세포를 주입하고 배양한 결과 대부분의 세포들이 융털 구조의 표면이 아닌 소자의 바닥 면에 부착하여 증식하는 것을 확인하였는데, 이는 수화젤 융털 구조에 비해 소자의 표면적이
상당히 크기 때문에 세포들이 상대적으로 소자의 표면에 많이 부착되어 발생한 것으로 파악된다. 미세유체소자 내 세포 배양 조건을 최적화 한다면 더욱 실제 체내 환경과 유사한 장내 미세 환경을 모사할 수 있을 것으로 기대된다. 본 연구에서 제안한 수평 방향 공정 방법을 이용하면 기존의 장내 환경 연구에 사용되었던 공정 방법에 비해 다양한 모양의 융털 구조를 손쉽게 제작할 수 있다. 또한 수화젤 혼합물의 조성을 바꾸어줌에 따라 물리적, 생리적 변화를 줄 수 있어 다양한 형태의 수화젤 구조와 결합하여 질병 모형 모사 및 개인 맞춤형 연구를 진행할 수 있을 것으로 전망된다. 그리고 유체 채널과 결합된 플랫폼의 장점을 이용하여 채널 내의 수화젤 구조물에서 배양되는 세포들이 다양한 방식으로 조절된 유체에 따른 반응을 확인할 수 있을 것이다. 마지막으로 본 연구에서 제안한 플랫폼은 다른 생체 모사 연구뿐만 아니라 체외 약물 검사, 은간온어침(Organ on a chip) 등의 연구에도 다양하게 적용될 수 있을 것이다.
References


