인체 장상피세포의 체외배양 및 실시간 관찰용 하이드로젤 기반 미세유체소자

Hydrogel Incorporated Microfluidic Device for In Vitro Culture and Dynamic Observation of Human Intestinal Epithelial Cells

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Human Intestinal Epithelial Cells

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KAIST

A thesis submitted to the faculty of KAIST in partial fulfillment of the requirements for the degree of “Master of Science”. The study was conducted in accordance with Code of Research Ethics

2013. 6. 14

Approved by

Professor Je-Kyun Park

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이 영

위 논문은 한국과학기술원 석사학위논문으로 학위논문심사위원회에서 심사 통과하였음.

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To complement limitations of the traditional methods for intestinal microbiota researches, a biomimetic in vitro model is demanded. Microfluidic models have been developed to meet such demand, but even they had to be sliced into thin sectional samples in order to observe microbiota environment such as epithelial monolayer, mucus and microorganisms. This study aims to develop a thin-section, microfluidic model allowing dynamic, simultaneous observation of microbiota environment. 20 μm thin collagen scaffold was constructed inside the microfluidic device as a barrier separating apical from basal flow and extracellular matrix (ECM) for epithelial culture. The collagen scaffold successfully allowed adherence of epithelial culture consisted of HT-29 intestinal cell line. Seeding density and cell incubation time suitable for covering the collagen scaffold with monolayer culture of epithelial cells were determined. HT-29 cells were found to secrete more mucus, which provides microbiota niche, when adhered to collagen. By quantifying mucus secretion of the HT-29 cells cultured inside the proposed device, the increased mucus secretion was found to be more dependent on cell—ECM adhesion than cell—cell adhesion. Dynamic, simultaneous observation of the collagen scaffold, the cell culture and green fluorescent protein (GFP)-expressing bacteria on single focal plane was demonstrated using the microfluidic device. During three days of cultivation, HT-29 cells displayed excessive proliferation, requiring inhibition of their tumorous phenotype. The proposed device offers a capability for live imaging of intestinal microbiota and its surroundings. Findings of this thesis study will be implemented to improve the device as truly biomimetic modeling of human intestinal microbiota.
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<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>dH$_2$O</td>
<td>deionized water</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>LB</td>
<td>Luria broth</td>
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<tr>
<td>L/D viability stain</td>
<td>LIVE/DEAD® Viability/Cytotoxicity kit</td>
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<tr>
<td>MEM</td>
<td>minimum essential media</td>
</tr>
<tr>
<td>MTX</td>
<td>methotrexate</td>
</tr>
<tr>
<td>PAS stain</td>
<td>Periodic acid–Schiff kit</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PDMS</td>
<td>poly(dimethylsiloxane)</td>
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<tr>
<td>PEG</td>
<td>poly(ethylene) glycol</td>
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<td>PLL</td>
<td>poly-L-lysine</td>
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1. Introduction

1.1 Importance of Researching Intestinal Microbiota

Microbiota is a huge population of microorganisms living inside host organisms. Host induces selection pressure on microbes, and subsequently emerged properties of microbiota affect host fitness; and this coevolutionary feedback gives rise to host specificity of microbiota [1, 2]. Numbers of microbiota and their genes far exceed those of cells composing the host. For example, microbes dwelling in human intestine surpass more than 100 trillion cells and may contain more than a hundred times genes in human genome [1, 2]. A few thousands of species have been identified in the intestinal microbiota, and 70% to 80% of the trillions of bacteria cannot be cultured in vitro [3]. Not only the shear population, genetic diversity and undiscovered identities of microbiota, but their interaction with host made microbiota a pursued issue in biological studies.

1.2 Conceptualization of Intestinal Microbiota, and Correlation between Microbiota and Human Health

Because the intestine is the biggest microbiota habitat in human body [1], intestinal microbiota has been extensively studied. Microbiomic niche inside gastrointestinal system was discovered to be the surface and inside of mucus layer secreted by epithelium (Figure 1) [4-6]. It was found that gastrointestinal microbiota control digestion, metabolism and inflammation [3]. An in vitro culture study found probiotic Lactobacillus GG upregulating intestinal mucus secretion and preventing pathogenic Escherichia coli (E. coli) adhering to mucosa [7]. Microbial genomics studies discovered obese patients having a population of
microbiota specific to obesity [8]. Bacterial infection of the epithelium and mesenchymal tissue was found to be associated with intestinal diseases, especially 20% of all malignant cancer [9].

**1.3 In vivo Models for Intestinal Microbiota Studies**

Limited accessibility to human gastrointestinal system has been a major challenge against invasive, *in vivo* studies on human. Therefore, animal models have been extensively used to study intestinal microbiota. Histochemistry on a fixed thin-section sample is a traditional research tool, and an extremely sensitive method when coupled with immunofluorescence technique [10]. As accurate measurement of such small entities on a thin-section as thin as 10 to 100 μm is possible, histochemistry led to scientific findings on detailed information of intestinal microbiota. Topological difference in microbial interactions at various locations of intestinal tissue was found in a study utilizing zebrafish model [11, 12]. Outstanding sensitivity of immunohistochemistry was essential for Johansson *et al.* who discovered that
intestinal mucus is composed of an underlying dense layer and a light layer, which harbors microorganisms, on top of it [13]. Live, persistent observation of animal microbiota was realized [14] in spite of limited resolution. Sadly, both methods were limited by the inherent nature of animal studies: scientific findings could not be directly implemented on phenomenon occurring in human microbiota due to host specificity.

1.4 In vitro Microfluidic Models for Intestinal Microbiota Studies

Demand for an in vitro biomimetic model of human intestinal epithelium has arisen to resolve limits of traditional methods for intestinal studies [5]. Microfluidics has shed a light on advancing in vitro models because biomimetic characteristics could be mimicked with accurate controllability of microfluidic technology. Imura et al. integrated Transwell®, which is a conventional in vitro model system for evaluating intestinal absorption, with microfluidic perfusion [15]. The flow conditioned intestinal cell culture with biomimetic shear stress so that drug absorption rate of the culture was more comparable to in vivo value. Kim et al. developed “Gut-on-a-chip” which induced biomimetic strain and shear stress on in vitro gut epithelium grown on plastic porous membrane [16]. Juxtaposing vacuum chambers stretched the porous membrane in order to induce biomimetic strain on the cell culture, thereby dramatically increasing its epithelial phenotypes. Another notable study fabricated biomimetic intestinal villi with collagen type I [17]. The villi structures with 200 μm diameter and 600 μm height matched the dimension of actual human intestine villi, but were not integrated to flow system.
1.5 Thin-section, Lateral Observation of Microbiota Environment

Lab-on-a-chip technology has been demonstrated for *in vitro* biomimetic model of human intestinal epithelium. In aforementioned lab-on-a-chip technologies, microbiota niche would form along the z-axis of (perpendicular from) microscopic observation planes, and thus the entire depth of the niche could not be observed on a single x-y axis focal plane. The explained models had to be sliced into thin-section samples to observe microbiota environment in a single focal plane.

Thin-section sampling has been frequently used in *in vivo* and *in vitro* researches for lateral observation of microbiota environment. Mucosal stratification and topology of microbiota were discovered from investigating thin-section samples from *in vivo* studies [12]. Phenotypes of intestinal epithelium such as mucus secretion and microvilli on apical face of cell monolayer were observed from thin-section samples in 2D *in vitro* models [18, 19]. From imaging of thin-section slices, the cell culture of “gut-on-a-chip” was discovered not to be squamous but polarized monolayer resembling intestinal epithelium [16].

Thin section slicing would not be required if a model were designed to contain entire microbiota environment within a single x-y axis focal plane. Constituents of microbiota environment (bacteria, mucus, epithelium, and interstitial matrix) would be observed laterally without destroying the model system. Persistent, lateral observation of thinly spread intestinal model will promise an effective approach for researching cell–bacteria interaction of intestinal microbiota.
1.6 Research Objectives

With a grand goal of developing an in vitro microfluidic model for researching intestinal microbiota, this study aims to fabricate a thin-section model for observing entire microbiota environment, which are microbiota, mucus layer, epithelium and interstitial matrix, in single focal plane simultaneously and persistently. A microfluidic device can be fabricated to specific dimension, and its height will match the typical thickness of a thin-section sample. It will be designed to accommodate thin intestinal epithelium culture of which side profile will be observed. Hydrogel structure will be constructed in the middle of a wide microchannel as a permeable barrier and scaffold for HT-29 epithelial cell line. Two kinds of fluids will be injected to the microchannel through different inlets, and separated from each other by the hydrogel structure. Injecting cell suspension in one of the two flows will allow adhesion of mammalian cells on one facade of the hydrogel structure.

HT-29, a cell line originating from human intestinal epithelial cell, will be seeded on the scaffold and have their morphology observed. Seeding condition of HT-29 cells will be optimized in order to maintain cell culture that well adheres on the hydrogel surface as a monolayer, not a lump of cells piled on top of each other. Persistent monitoring of the cell culture will be demonstrated in the proposed device. Mucus production of the cell culture will be examined to study the effect of cellular adhesion on hydrogel surface. Fluorescent bacteria will be inoculated on the cell culture, and brightfield and fluorescent microscopy will be utilized to demonstrate persistent, simultaneous observation of all components of the thin-section microbiota model—ECM, cell and microbiota.
2. Materials and Methods

2.1 Design Criteria

As intestinal epithelium exchanges nutrients with mesenchymal tissue in basal region, and interacts with microbiota in apical region, the proposed microfluidic device must allow apical and basal perfusion with epithelial culture sandwiched between them. Hydrogel scaffold which is situated between apical and basal perfusion channels requires two roles. The scaffold must act as a permeable barrier separating the two flows to prevent migration of particles as big as epithelial cells while allowing exchange of nutrients. Such barrier must have molecular sites on which epithelial cells can adhere, thus providing mesenchymal environment. Preliminary experiments of constructing the scaffold with alginate or poly(ethylene) glycol (PEG) was dissatisfactory. Biblical researches were done to find more suitable hydrogel material.

Interstitial matrix, which provides structural support and niche for mesenchymal cells, of intestinal epithelium is primarily composed of collagen type I [20]. Between epithelial cells and the matrix exists 50–100 nm thin interface called basement membrane (BM) (Figure 2). Therefore, Matrigel™ and collagen type I were chosen among the candidates (Table 1), and methods to construct hydrogel scaffold as ECM within the microfluidic device was developed.
Figure 2. Illustration of epithelial and mesenchymal compositions of intestinal tissue.

Table 1. List of candidate materials for the hydrogel scaffold.

<table>
<thead>
<tr>
<th></th>
<th>Alginate</th>
<th>PEG</th>
<th>Matrigel™</th>
<th>Collagen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structure</td>
<td><img src="image" alt="Alginate" /></td>
<td><img src="image" alt="PEG" /></td>
<td>Mixture of serum-derived proteins from basement membrane</td>
<td><img src="image" alt="Collagen" /></td>
</tr>
<tr>
<td>Pros</td>
<td>Easy handling</td>
<td>Excellent patterning</td>
<td>Mammalian cells do adhere</td>
<td>Mammalian cells do adhere</td>
</tr>
<tr>
<td>Cons</td>
<td>Mammalian cells do not adhere</td>
<td>Mammalian cells do not adhere</td>
<td>Flimsy interface protein</td>
<td>Handling difficulty</td>
</tr>
</tbody>
</table>
Aiming for developing an *in vitro* model for researching intestinal microbiota, this study proposes a thin-section model allowing persistent, lateral observation (Figure 3). Therefore, height of the hydrogel scaffold should approximate 20 μm, which is comparable to thickness of a thin-section tissue sample. Mammalian cells would adhere on the sides of the hydrogel scaffold which is exposed to apical and basal flow (Figure 3). As typical diameter of a mammalian cell ranges from 10 to 20 μm, one or two layers of biomimetic epithelium are expected to form on the scaffold.

**Figure 3.** Depictions of the design criteria: hydrogel incorporation (a); and thin-section lateral observation (b).

### 2.2 Device Schematics

To construct the middle barrier with hydrogel material, “surface-tension-confined microfluidics” was applied [21, 22]. The proposed device has a dual layer microchannel with a narrow gap in the middle (Figure 4). Three subchannels—cell suspension, hydrogel and media—were distinguished in the microchannel. If hydrogel material was injected to the hydrogel subchannel, the material would only fill it without flooding the juxtaposed subchannels thanks to capillary action (Figure 4).
Width of the hydrogel scaffold was set to 200 μm because it is the efficient diffusion limit of small molecules in mammalian tissue [23]. Channels #1 and #2 were 2400 μm wide in order to accommodate the widest mucus layer measured in vivo, 800 μm [6], and reserve ample media in each channel to improve cell survival during seeding. The 20 μm high hydrogel scaffold would accommodate 1 to 2 layers of mammalian cells (Figure 4), and the juxtaposing channels were 60 μm as this height ratio was found to ensure selective filling of the narrow middle channel [22].

**Figure 4.** Design schematics of the thin-section microfluidic device.
Because the hydrogel subchannel was merely 20 μm tall, it could be easily deformed and clogged by gravitational force and fluidic pressure. Vertical deformation of PDMS wall was found prevalent in very shallow channels with huge width (Figure 5) [24]. Air bubbles could be trapped inside the microchannels, and pressure was given inside the microchannels in order to drive out the bubbles through gas-permeable PDMS wall [25].

**Figure 5.** Pressure-driven air bubble removal inside PDMS microchannel (a) caused bulging of the channel wall when the channel width (W) was far greater than the height (h) (b). Incorporation of PDMS micropillars in the middle channel mitigated bulging in the region (c).
When the hydrogel channel is expanded upward, the adsorption between hydrogel and PDMS surface would be broken. PDMS micropillars with 20 μm diameter were built in the hydrogel channel to prevent the deformation (Figure 6). Without the pillars, the hydrogel channel bulged by 20 μm. Even with the least numbers of the pillars, the deformation was halved. Although the most densely arranged micropillars mitigated the deformation most, they also hindered hydrogel injection. Therefore, Type 3 and 4 were favored for conducting subsequent experiments. It was also postulated that the adsorption between hydrogel scaffold and PDMS surface would be enhanced thanks to increase in the surface area of PDMS in the middle subchannel by the incorporation of the micropillars. Figure 7 illustrated the final design schematics.

(a)  
(b)  

**Figure 6.** Measurement of the hydrogel channel bulging (Δh) and the mitigating effect of the PDMS micropillars (a); and various arrangements of the PDMS micropillars (b). Type 1 represented absence of the PDMS micropillars.
Figure 7. Design schematics of the thin-section microfluidic device with the micropillars.
2.3 Materials

Silicon wafers were purchased from Helitek Co. Ltd. (Fremont, CA) and SU-8 2025 negative photoresist from Microchem (Newton, MA). Sylgard® 184 of Dow Corning (Midland, MI) was obtained for poly(dimethylsiloxane) (PDMS). 0.01% aqueous solution of poly-L-lysine (PLL) was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO). Matrigel™ and high concentration collagen type I from rat tail were obtained from BD Biosciences (Bedford, MA). NaOH and phosphate-buffered saline (PBS), 10X concentration, were purchased from Junsei (Tokyo, Japan) and Invitrogen (Carlsbad, CA), respectively. For bead-based experiment, Fluoresbrite® 6.0 μm diameter green fluorescent microspheres from Polysciences, Inc. (Warrington, PA) were used. HT-29 colorectal adenocarcinoma and human umbilical vein endothelial cells (HUVEC) were bought from the Korean Cell Line Bank (Seoul, Korea). E. coli expressing the green fluorescent protein (GFP) was kindly provided by Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). LIVE/DEAD® Viability/Cytotoxicity Kit (L/D viability stain) from Invitrogen (Carlsbad, CA) and Periodic acid–Schiff (PAS stain) kit from Sigma-Aldrich were utilized. Chamlide top-stage incubators for microscope—manufactured by Live Cell Instrument (Seoul, Korea)—allowed persistent observation of cell culture. Zeiss AxioCam ICm1 microscope camera from Carl Zeiss Microscopy GmbH (Jena, Germany) and Olympus DP72 microscope camera from Olympus (Tokyo, Japan) were used for image acquisition. A PHD 2000 syringe pump was purchased from Harvard Apparatus (Holliston, MA).
2.4 Microchannel Fabrication

A microfluidic device was fabricated via double-step soft lithography of PDMS (Sylgard 184; Dow Corning, Midland, MI) molding process (Figure 8). To make the mold of the device, the negative photoresist SU-8 2025 (MicroChem Corp., Newton, MA) was spin-coated and lithographed on a bare silicon wafer. The elastomer was mixed with a curing agent in a ratio of 10:1 (w/w). PDMS was cast on the mold and cured for 3 h in a convection oven at 65 °C for complete cross-linking. The replicates and flat PDMS foundations were plasma-activated and bound together after exposure to oxygen plasma for 30 s.

**Figure 8.** Fabrication process of the microfluidic channel and hydrogel scaffold.
2.5 Hydrogel Scaffold Fabrications

A completed device was sterilized with an injection of 70% ethanol and dried. For constructing Matrigel™ scaffold, a sterile device was stored in a humidified 37 °C incubator to saturate the inside with moisture, and then cooled to 4 °C. Matrigel™ stock, defrosted to 4 °C, was injected to the hydrogel loading inlet while the cold temperature was maintained in ice bath. The device was immediately stored in the humidified incubator for 15 min to polymerize the Matrigel™ injection (Figure 8).

For constructing collagen scaffold, the capillary of a sterile device was coated with PLL solution as PLL is known to promote binding between collagen and PDMS surface [26]. The PLL filled device was slowly dried for 3 h at 37 °C. The dried device was cooled to 4 °C for 15 min. Meanwhile, high concentration collagen type I in ascetic acid solution was neutralized with 0.1 M NaOH and 10× PBS: the composition was 17 parts collagen, 1 part 0.1 M NaOH and 2 parts 10× PBS. The mixing was done in ice bath to maintain 4 °C temperature. The chilled, neutral collagen solution was injected to the hydrogel loading inlet while the cold temperature was maintained in ice bath. The device was immediately stored in a humidified 37 °C incubator for 30 min for polymerization (Figure 8).
2.6 Bead-based Leakage Test of Hydrogel Scaffold

Upon filling the hydrogel subchannel with hydrogel, 6.0 μm diameter green fluorescent microspheres were apically perfused. Fluorescent images were taken to observe whether the beads penetrated the hydrogel (either Matrigel™ or collagen) barrier and diffused into the basal subchannel.

2.7 Batch Cell Culture

HT-29 is a cell line originating from colorectal adenocarcinoma. It has been favored for \textit{in vitro} researches on intestinal mucosa studies because its originating primary cell is mucus secreting intestinal goblet cell [27]. HUVEC was chosen as negative control for mucus secretion. Both HT-29 and HUVEC were grown in separate BD Falcon™ 25 cm$^2$ flasks to 80% confluency. Minimum essential media (MEM) supplemented with 10% bovine FBS and 100 mg/L ampicillin and EGM™-2 media were supplied to each culture. GFP-expressing \textit{E. coli} was cultured in Luria Broth (LB) supplemented with 100 mg/L ampicillin at 37 °C.
2.8 In-chip Cell Seeding

The mammalian cells were collected via trypsinization, and their numbers were counted with hemocytometer. Counted cells were injected in the apical side of the gel-filled device. The device was incubated upright—the device being perpendicular to the floor—in a humidified 37 °C incubator to allow cells to settle down and touch the hydrogel scaffolds (Figure 9).

Figure 9. Process for culturing mammalian cells and inoculating bacteria inside the microfluidic device.
2.9 In-chip Cell Culture Perfusion Setup

With cells adhered on the hydrogel scaffold, the device inlets and outlets were connected to media reservoir and Harvard syringe pump, respectively, via Tygon® tubing. The device was maintained in a Chamlide top-stage incubator which was installed on a microscope with Zeiss Axiocam ICm1 camera for persistent observation (Figure 10). The perfusion system provided fresh media and allowed staining agents to be introduced to in-chip cell culture.

Figure 10. Experiment setup for in-chip cell cultivation and media perfusion.
2.10 Characterization of In-chip Cell Culture

HT-29 cells were seeded in different concentrations (6.5×10^5 cell/mL, 1.8×10^6 cell/mL, and 6.5×10^6 cell/mL) to determine suitable seeding cell number for establishing biomimetic intestinal epithelium. The condition of each culture was characterized with the percentage of hydrogel scaffold covered with adhered cells and the width of the cell culture layer. Various incubation periods (0, 11, and 20 h) for seeded cells were tested in order to select the most suitable time for constructing the epithelium.

2.11 L/D and PAS Staining

PBS injection on both apical and basal inlets washed cell culture inside the device. The L/D stain was injected apically and kept for 30 min to stain the cell for viability measurement. PAS staining was completed in similar manner. The cells were washed with deionized water (dH₂O), treated with periodic acid for 5 minutes, washed with dH2O, Schiff’s reagent for 15 min, and then washed with dH₂O. Fluorescent images of the L/D stain and the visible colorimetric images of the PAS stain were obtained with Olympus microscope.
2.12 PAS Intensity Quantification

Colorimetric PAS intensity of the stained human cells was quantified with ImageJ software [28]. All images were converted into black and white. An arbitrary threshold value was implemented to visualize only the PAS intensity stronger than the cutoff. Cellular PAS intensity was quantified in following culture conditions—2D culture, 2D culture on collagen coated surface, and in-chip culture.

2.13 Bacterial Inoculation

LB culture of GFP-expressing bacteria was diluted with the supplemented MEM, and injected to the apical channel of the device where HT-29 was being cultured (Figure 9). Fluorescent and brightfield images were taken at different time points—0 and 4 h from the point of perfusion—with a fluorescence microscope.
3. Results and Discussion

3.1 Bead-based Leakage Test of Matrigel™ and Collagen Scaffolds

To check sealing capability of the hydrogel barrier, 6 μm diameter green fluorescent microbeads were injected to the apical subchannel of the microfluidics device. The 6 μm diameter was chosen because it represented the diameter of trypsinized mammalian cells which would be loaded to the device. If the beads were penetrating the hydrogel structure, mammalian cells, etc., would penetrate instead of adhering on the surface and forming epithelium culture. Figure 11 showed that the microbeads could not penetrate both Matrigel™ and collagen scaffold but pile on the exposed surface. However, the Matrigel™ scaffold dried during the 30 min polymerization process while the collagen sustained minimal damage (Figure 12). If HT-29 cells were seeded on the surface of each hydrogel structure, they would attach on the surface instead of invading the internal space. However, only on the undamaged collagen scaffold would they form a well oriented linear culture. The results displayed that collagen had superior structural property as the hydrogel scaffold. In addition, stacking of the fluorescent bead suggested seeding concentration must be controlled to prevent overloading of mammalian cells.
Figure 11. Leakage test with 6 μm diameter fluorescent beads on; (a) Matrigel™; and (b) collagen scaffolds.

Figure 12. Dried hydrogel scaffold—(a) Matrigel™ and (b) collagen—after 30 min polymerization process.
3.2 Comparison of Epithelial Cell Attachment on Matrigel™ and Collagen Scaffolds

Trypsinized HT-29 cells (1.8×10⁶ cell/mL) were injected to the apical subchannel, and the device was incubated upright in static condition for 20 h to let the cells settle down and attach. On Matrigel™, HT-29 did not attach and retained spherical morphology while attaching on collagen and showing epithelium like formation (Figure 13). The result corresponded with a research which found HT-29 cells to attach better on collagen type I than basement membrane proteins [29]. Showing superior cellular property on top of aforementioned structural durability, collagen was selected as suitable hydrogel material for the microfluidic model.

Cells were found infiltrating the Matrigel™ scaffold (Figure 13). It signified that Matrigel™ adsorbed on PDMS surface of the hydrogel subchannel was broken off, and cells were infiltrating the interface of hydrogel and PDMS. The hydrogel subchannel was prone to bulging because less PDMS micropillars were embedded. Densely placed micropillars, as shown as Type 3 and 4 in Figure 6, were essential for preventing cells from infiltrating the hydrogel scaffold by minimizing the breakage of hydrogel scaffold from PDMS surface.
Figure 13. Cultivation of HT-29 cells in the microfluidic device when the hydrogel material is Matrigel™ (a), or collagen (b). HT-29 cells only adhered to collagen.
3.3 Measuring the Effect of Collagen Scaffold on Cellular Mucus Production

The effect of cellular attachment on collagen on mucus production was investigated with PAS staining. PAS stains cellular carbohydrates at neutral pH and is commonly used for detecting mucus, because the major component of mucus is a family of heavily glycosylated proteins called mucins [18, 6]. Cells were cultured on bare 2D plastic plates or on plates coated with collagen type I. HUVEC cells were cultured, too, as a negative control against HT-29. PAS staining was conducted 20 h after seeding. HT-29 grown on collagen surface secreted twice the amount of mucus compared to ones grown on uncoated surface (Figure 14). Negative control of HUVEC showed six-fold increase when grown on the collagen substrate, but the PAS intensity was remarkably lower than the cases of HT-29 (Figure 14). Cellular adherence on collagen, which signified cell to ECM interaction, was found to enhance mucus secretion. This finding emphasized the importance of ECM scaffold in establishing human intestinal model for microbiota studies. When grown on artificial, plastic surface, human intestinal epithelial cells would not be able to secrete ample mucus as viable niche for intestinal microbiota.
Figure 14. Quantitative comparison of cellular mucus production by PAS intensity. HT-29 cells produced larger amount of mucus. Adhesion on collagen upregulated mucus production.

3.4 Establishing the In-chip Culture Condition: Cell Seeding Concentration

Various HT-29 seeding concentration was tested to find the most suitable concentration for culturing HT-29 cells in the microfluidic device. Seeding procedures were done in three different concentrations—$6.5 \times 10^5$ cell/mL, $1.8 \times 10^6$ cell/mL, and $6.5 \times 10^6$ cell/mL. The devices containing HT-29 cells were incubated in static condition for 1 day, and then bright field images were taken (Figure 15). With the seeding concentration of $6.5 \times 10^5$ cell/mL, adhered cells were sparse and retained spherical morphology. Although HT-29 cells were cultured as a monolayer, the culture was not comparable to intestinal epithelial monolayer in vivo due to the undifferentiated morphology. Most of the scaffold was covered with single to triple layers of HT-29 cells when the condition was $1.8 \times 10^6$ cell/mL. Regions of well adhered
monolayer culture were observed in the device at this concentration. Although the entire collagen scaffold was covered with HT-29 cells when the seeding concentration was $6.5 \times 10^6$ cell/mL, excessive cells were seeded. The concentration of $1.8 \times 10^6$ cell/mL was deemed suitable for the in-chip cell culture providing excellent cellular adhesion and regions of monolayer culture while the rests were either underloading or overloading conditions.
Figure 15. Differences in culture morphology resulted from various cell seeding concentrations: (a) $6.5 \times 10^5$ cell/mL; (b) $1.8 \times 10^6$ cell/mL; and (c) $6.5 \times 10^6$ cell/mL.
The seeding concentrations were assessed numerically with two properties—coverage percentage and culture width (Figure 16). Coverage percentage was the proportion of the collagen structure covered by attached HT-29 cells. Culture width was an average spread of cell culture from surface of the collagen scaffold. 54.7%, 93.9% and 100% of the collagen structure was covered with HT-29 at the seeding concentrations of $6.5 \times 10^5$ cell/mL, $1.8 \times 10^6$ cell/mL, and $6.5 \times 10^6$ cell/mL, respectively. Sections of 16 μm wide monolayer were sparsely formed with the smallest seeding concentration. Seeding at $6.5 \times 10^6$ cell/mL resulted in average culture width of 76.6 μm, and thus multilayered swarm of HT-29 was cultured instead of a monolayer. The average culture width was 39.3 μm in the moderate seeding density. Mono-, double- and triple- layers of HT-29 were cultured in this condition as displayed by the range of standard deviation. The $1.8 \times 10^6$ cell/mL was an appropriate condition to observe monolayer in the microfluidic device.

![Figure 16](image_url)

**Figure 16.** The adhered cell coverage percentage and the culture width were measured at different seeding densities. A graph plot (a) and illustrations of coverage % and culture width (b) are presented.
3.5 Establishing the In-chip Culture Condition: Static Incubation Time and Viability

The time needed for the seeded HT-29 cells to adhere on the collagen scaffold was found. HT-29 cells were injected to the microfluidic device at the concentration of \(1.8 \times 10^6\) cell/mL. The device was kept upright from the floor to let cells settle down and contact the hydrogel surface. Condition of in-chip cell culture was checked at multiple time points during static incubation (Figure 17). The 0 h image was taken 30 min after keeping the device upright. At 0 h, most cells were clumped together and set apart from the hydrogel surface. Up to 11 h, HT-29 cells kept spherical morphology. However, most cells were found contacting the hydrogel surface except regions on which a group of cells were stacked double or triple layers. Although the image was taken at same region, the number of cells decreased at 11 h because unattached cells floated away from the viewpoint when the upright device was flipped belly down on the microscope stage for imaging. It was at 20 h during which HT-29 attached to the hydrogel surface and tightly to each other, hence losing the spherical morphology. Approximately one full day of static incubation of HT-29 in the microfluidic device was necessary for them to adhere on the scaffold surface. Cell viability after the incubation was 97.2%. Detached, yet viable cells in Figure 17d were transported to the viewpoint from other regions of the microfluidic device by the perfusion of L/D viability stain. Media contained in the cell suspension which was injected to the 2400 μm wide channel was enough to nourish cells until they adhere.
Figure 17. Differences in culture morphology resulted from various incubation time: (a) 0 h; (b) 11 h; and (c) 20 h. Cellular adhesion was observed after 20 h of incubation after seeding. Viability of the culture was measured 97.2% after the 20 h incubation (d).

3.6 Quantifying Cellular Mucus Production in the Microfluidic Model

To distinguish which interaction was encouraging mucus secretion, the amount of cellular mucus production in the microfluidic model was measured as PAS intensity per cell in two regions where the cells were bound to collagen (Cell–ECM) or themselves (Cell–Cell). The Cell–ECM region was overlaid 30 μm from the surface of collagen scaffold, and the Cell–Cell region was 30 to 80 μm (Figure 18). Role of the collagen scaffold as intestinal ECM would be clarified if HT-29 cells behaved differently when interacting with collagen or other HT-29 cells. HT-29 cells in the Cell–ECM region expressed PAS intensity four times more than those in the Cell–Cell. Increase in mucus production was dependent on cell to ECM ad-
hesion, not cell to cell. Hence, the collagen scaffold was shown to be an essential component in this study to induce *in vitro* human intestinal epithelial tissue to secrete mucus layer wide enough to provide microbiota niche and protect epithelial culture from luminal flow. The HUVEC cells expressed about one third of PAS intensity than HT-29. HUVEC cells were only counted in the Cell–ECM region because of light seeding concentration (8.0×10^4 cell/mL) and non-specific adherence on PDMS surface. The comparison highlighted superb mucus production of HT-29 cells, which displayed a potential to create microbiota niche in the microfluidic device.

![Image](a) (b)

**Figure 18.** Cellular mucus production (PAS intensity/cell) in the microfluidic device was measured in the Cell–ECM and Cell–Cell regions (a), and documented in a bar graph (b).
3.7 *E. coli* Inoculation

This research set a goal to fabricate a microfluidic device able to observe the entire microbiota environment in single focal plane without killing the sample. GFP-expressing *E. coli* was inoculated to an in-chip HT-29 culture to demonstrate the claimed observation method. In this experiment, bacteria, HT-29 and ECM were observed simultaneously in single focal plane (Figure 19). Presence of the epithelial culture deterred bacteria from contacting surface of the collagen structure. Bacteria were contacting periphery of the culture instead. Fresh media was perfused at 150 $\mu$L/h for 4 h immediately after the inoculation. Images were taken at the same region to observe any changes in the culture. Morphology of the HT-29 culture changed, showing cells proliferating away from the collagen scaffold. All but one bacterium were washed off from the surface of the culture. From the elimination of the bacteria, it was postulated that a period of static incubation would be necessary to have bacteria attach on the surface of HT-29 cells and be able to withstand media perfusion. This demonstration—consistent observation of an *in vitro* culture without fixation—provides an opportunity to observe the thin-section microbiotic environment model *in situ*.
Figure 19. Images of GFP-expressing *E. coli* inoculation on the in-chip HT-29 culture were taken immediately after the inoculation (a), and after 4 h of fresh media perfusion at 150 μL/h (b). Perfusion was initiated immediately after the inoculation.

### 3.8 Proliferation Behavior in 3 Day In-chip Culture

Proliferation behavior of HT-29 culture differed by initial seeding concentration (Figure 20). 2 day long in-chip culture was conducted after a full day of static incubation with media flowing in both apical and basal channels. The device was kept in Chamlide top-stage microscope incubator and connected to perfusion system for persistent observation (time lapse recordings of HT-29 proliferation). When seeded in low density, HT-29 cells did not adhere on the collagen surface. Cells did not proliferate along the surface of the collagen scaffold, but dislodged from their original locations by perfused media. On the other hand, densely seeded HT-29 cells showed excessive growth. Growth profile of HT-29 culture was
postulated to be related to initial seeding concentration.

HT-29 cells were found infiltrating the collagen scaffold regardless of the initial seeding concentration. Their un-polarized morphology indicated they were advancing through the gap between collagen and PDMS surface. The collagen scaffold could easily be detached from PDMS surface, and cells would accelerate the detachment by invading the weakest spot of the collagen scaffold—the interface between collagen and PDMS. It is strongly recommended to immobilize the collagen scaffold to PDMS surface based on this observation.

**Figure 20.** Proliferation of the in-chip HT-29 cultures over 3 days. Seeding densities were: $6.5 \times 10^5$ cells/mL for (a) and (b); and $6.5 \times 10^6$ cells/mL for (c) and (d). Media was perfused for the last 2 days.
4. Conclusions

4.1 Summary

This study aimed to develop a novel microfluidic model of human intestinal tissue by dynamically observing whole tissue model on single focal plane. A microfluidic device for \textit{in vitro} culture and dynamic observation of the human intestinal tissue and microbiota niche, which was consisted of ECM, epithelial cell and microbiota, was fabricated. A dual layer microchannel consisted of a narrower capillary sandwiched between two taller subchannels realized formation of 20 \( \mu \text{m} \) thin hydrogel barrier which served as ECM for the thin-section human intestinal model. Collagen type I was evaluated as a suitable hydrogel material, permitting HT-29 cells to attach. Appropriate seeding density and incubation period for establishing the epithelial culture was determined. HT-29 secreted ample amount of mucus and was suitable cell line for \textit{in vitro} human intestinal culture. Cellular adherence on collagen rather than cell—cell was discovered to increase mucus production. Dynamic, simultaneous observation of viable ECM, cell and microbiota in single focal plane was demonstrated in the proposed device. Culturing HT-29 cells inside the microfluidic device for 3 days indicated tumorous proliferation and the need to anchor the collagen scaffold to PDMS surface of the capillary subchannel. This research was a rudimentary demonstration of forming and dynamically observing human intestinal microbiotic environment in the fabricated device.
4.2 Future Direction

Subsequent improvements must be achieved in order to conduct advanced studies on this device. Cells infiltrating the boundary between collagen and PDMS (Figure 13 and 20) suggested sealing between collagen and PDMS was imperfect despite PDMS micropillars were incorporated. Chemical treatments for covalent binding of nanometer-thin film of collagen on PDMS surface [30, 31] can be utilized to immobilize the collagen scaffold in the proposed device. The improved device will allow mimicking interstitial flow in human intestinal tissue with no cellular infiltration. Width of the collagen scaffold will be adjusted to better reflect actual diffusion rate of human intestinal epithelium. Computational fluid dynamics will be a great aid in determining an optimized width.

When HT-29 was cultured in the device for 3 days, tumorous proliferation was observed (Figure 20). With current setup, long-term cultivation of a biomimetic epithelium is challenged due to tumorous proliferation of HT-29. HT-29 cells adapted to anticancer agent methotrexate (MTX) were found to differentiate and recover phenotypes of intestinal goblet cells [27, 19]. Cultivating MTX treated HT-29 cells is likely to refine biomimetic characteristics of the modeled epithelium and allow long-term culture. Adjusting the composition of apical and basal fluid will help improving the biomimetic characteristics. The 3-day culture experiment also showed importance of initial cell seeding concentration on establishing pseudo-epithelium. Besides optimizing the concentration, replacing the gravitational method with hydrodynamic approach will ensure culturing homogeneous monolayer of human epithelial cells.
Intricate researches will be done with further developed microfluidic system. Epithelial phenotypes will be explicitly examined in order to evaluate biomimetic capability of the in vitro intestinal tissue. In vitro studies of the interaction between host cells and communal or pathogenic microorganisms had been conducted on 2D cultures [32, 33]. Comparable researches accomplished on the microfluidic model presented in this study would better represent phenomena actually happening in human intestine. Because the in-chip HUVEC culture showed properties distinguished from 2D cultures, implementing the model system for studying other organs such as vasculature is proposed.
5. References

Summary in Korean

인체 장상피세포의 체외배양 및 실시간 관찰용 하이드로겔 기반 미세유체소자.

인간 장내 마이크로바이오타 연구를 위한 기존 기술의 한계를 극복하기 위해 생체모사성 체외모델이 요구된다. 이에 대한 해답으로 미세유체소자 기반 체외배양 모델이 제안되었으나, 기저조직, 상피세포, 점액, 미생물 등으로 이뤄진 마이크로바이오타 환경을 관찰하기 위해서는 위 모델을 얇은 절편으로 절단하여 표본을 획득해야 한다. 미세유체소자 모델 뿐만 아니라 동물 실험 및 기존 체외배양 모델 연구의 경우에도 마이크로바이오타 환경관찰을 위해 모델의 파손이 필수적이다. 위 문제를 해결하기 위해 본 연구에서는 마이크로바이오타 환경의 각 요소를 실시간 및 통시 관찰이 가능한 절편 형태의 미세유체소자 모델을 제안했다.

절편 두께의 인체 장상피조직모델의 기초가 되는 하이드로겔 기저층을 미세유체소자 내부에 형성하기 위하여 모세관현상기반 미세유체기술을 이용했다. 중앙구역의 높이가 20 μm 이며, 인접구역의 높이가 60 μm 인 복층구조의 마이크로채널을 설계했다. 중앙텐트로 주입된 하이드로겔 물질이 모세관현상에 의해 단차가 낮은 중앙구역에만 집중되어 위 마이크로채널을 두 개의 마이크로채널로 분리하는 구조물을 형성했다. 하이드로겔 구조물이 콜라겐으로 형성된 경우 HT-29 (cell line) 장상피세포가 당 구조물의 표면에 부착되어 성장했다. 또한, 콜라겐 표면에 부착된 장상피세포층을 배양하기 위해 적합한 세포시딩 농도 및 배양 시간을 확정했다. 콜라겐에 부착된 HT-29 세포가 더 많은 양의 점액을 분비하는 것을 관측했으며, 증가된 점액 분비량은 세포와
기저조직 간의 상호작용에 연관된 것으로 밝혀졌다. 제작된 미세유체기반 모델을 이용하여 콜라겐 구조물, 장상피세포, GFP 발현 대장균 등을 동일 조건상에서 실시간 관찰했다. 결과적으로 본 연구에서 목표한 기능인 마이크로바이오타 환경 구성 요소의 실시간 및 동시 관찰을 제작된 미세유체소자 모델에서 시연한 것이다. 본 연구에서 제안한 모델이 인간 장내 마이크로바이오타 연구에 큰 기여를 할 것으로 기대된다. 인체를 가복하여 장내 마이크로바이오타에 대한 연구를 수행할 수 없기 때문에 본 미세유체소자 모델에서 생체모사 조직을 배양하여 대체실험을 수행할 수 있다고 전망한다.

또한, 인간 생체모사 마이크로바이오타 환경을 제안된 미세유체소자 내부에 구성하는데 필요한 개선안을 본 연구를 통해 밝혀냈다. 콜라겐 구조물과 PDMS 표면 사이의 간격으로 배양되는 장상피세포가 침투하며, 종양성 성장하는 것을 3일간의 배양실험에서 관찰했다. 따라서, 콜라겐 구조물을 마이크로채널 표면에 화학적으로 고정하여 세포침투를 방지하고, 항암제 처리를 통해 HT-29 세포의 종양성 형질을 억제할 필요가 있는 것이다. 개선요소를 반영한 미세유체소자에서 실제 조직과 표현형질이 유사한 장상피조직을 배양하고, 이에 기반하여 생체모사 마이크로바이오타 환경을 구성할 수 있을 것으로 기대된다.