A Novel Microfluidic Intestine Model With An Extracelluar Matrix Membrane

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ABSTRACT. We have developed novel microfluidic devices that incorporate a micrometer resolution membrane that is synthesized out of rat-tail type I collagen. Lyophilized Type I collagen was sandwiched between the layers of PDMS that had been prepared by replica molding. The resulting device had two independent channels separated by the membrane. We characterized the microstructure of the membrane. Through transport studies, we found that mass transport of 40kDa dextran was an order of magnitude higher through the collagen membrane than through the Transwell membrane. Caco 2 cells were cultured in devices with no Transwell and ECM membrane. We found that caco 2 cells cultured on the collagen membrane had excellent viability and function for extended periods of time compared to the other two devices. Our results indicate a substantial improvement in establishing a physiological microenvironment for in vitro organs-on-chips.

Introduction

The intestine is essential for processing food and drugs, absorption of nutrients, and generation of waste;1–3 chronic intestinal ailments affect our overall physiological health.4–6 Inflammatory intestinal disorders (IBD), which affect the health and function of the gut, are believed to be caused by complex interactions among the different cells, cytokines, and the microbiota.7–11
Understanding the underlying mechanisms of gut disorders is necessary to develop effective treatment and prevention strategies. As gut disease etiologies are multifactorial, the use of appropriate animal models is essential. However, animal models are limited in their ability to mimic the human physiology and diseases, especially IBDs, because of the wide differences between animal and human colons and their respective microenvironments including the microbiota. For example, animal models often fail to predict human intestinal physiological responses to orally taken drugs\textsuperscript{12} or to constitute appropriate disease models related to human disease,\textsuperscript{13} and human microbiota–intestine models,\textsuperscript{14} and drug–drug interactions.\textsuperscript{15,16} For these reasons, it is necessary to develop models that can mimic the physiological functions of human intestine in vitro.\textsuperscript{17–21}

Most recently, knowledge derived from tissue engineering is increasingly applied in the development of micro-engineered models of human tissues and organs.\textsuperscript{22} These models, also called microfluidic devices, are being used as potential in vitro alternatives to animal models in simulating morphogenetic and pathogenetic processes, as well as drug screening platforms.\textsuperscript{23–25} Microfluidic devices are increasingly found in research centers, clinics, and hospitals, contributing as more accurate and powerful tools for studying drug delivery, monitoring specific analytes, and medical diagnostics.\textsuperscript{22} The devices often introduce the third dimension (3D) to the in vitro cell cultures and are related to the integration of tissue engineering with microfabrication and microfluidics,\textsuperscript{17,26–28} advances in this field are associated with the convergence of biology with engineering. Integration of cells cultured within the microengineered platforms is often referred to as “organs-on-chips.” An organ-on-a-chip is a microfluidic device that contains continuously perfused chambers inhabited by living cells that allow modeling of the in vivo environment and enable precise control of cells and fluids\textsuperscript{29–32} to
recapitulate the physiological and pathological conditions of complex tissues and organs. This new technology is permissible to high-resolution, real-time imaging and in vitro analysis of biochemical, genetic, and metabolic activities of living cells, which make them critical tools for finding functional properties, pathological states, and developmental studies of organs.

The microfluidic chips are commonly fabricated out of polydimethylsiloxane (PDMS), owing to the material’s chemical inertness and biocompatibility. The devices typically contain multiple layers comprising a top layer, a bottom layer, and a porous membrane that is sandwiched between the two layers. Due to the excellent oxygen permeability of PDMS, the cells can be either cultured on the bottom layer or on the membrane in the microfluidic devices. To study the diseases in the gut, several groups have created an in vitro model of the intestine, or gut-on-a-chip. Different versions of the devices contain various components of the microenvironment such as cells, perfusion, bacteria and inflammation, nutrients, and extracellular matrix (ECM). Shah et al. developed a modular microfluidics-based model, which allows co-culture of caco 2 colon cells with Bacteroides caccae and Lactobacillus rhamnosus GG under conditions representative of the gastrointestinal human–microbe interface and showed that the co-culture results in a transcriptional response, which is distinct from that of a co-culture solely comprising Lactobacillus rhamnosus GG. Using caco 2 cells, Kim et al. developed a gut-on-a-chip PDMS device and showed that the cells underwent villus differentiation, while co-cultures of caco 2 cells and a formulation of probiotic bacteria (VSL#3) containing eight strains of probiotic bacteria improved barrier function. Organs-on-chips are also widely used in infection, relation of nanoparticles and disease, and drug metabolism and screening. One common feature of all of these microfluidic organs is the use of synthetic membranes as the supporting material for cell growth. Often, these membranes have non-physiological pores spanning several micrometers and restrict movement of cells across the barrier. Cells grown on these polyester and polydimethylsiloxane (PDMS) membranes can develop unintended pathology; for example, gut
epithelial cells formed non-physiological squamous epithelium rather than the columnar forms observed in vivo. Further, the inherent high hydrophobicity of the synthetic surfaces affects cells’ viability, adhesion, and aggregation. To overcome these issues, the membranes have to be treated with plasma or surface coatings.

In this paper, we report the development and characterization of a multilayer microfluidic device that incorporates a novel membrane, which is made out of type I collagen. We selected type I collagen because of several reasons. Type I collagen is one of the most abundant proteins in the extracellular matrices in vivo and is widely used as a supporting matrix. Type I collagen is the primary constituent of the major ECM proteins in the intestine involved in several cellular processes including remodeling, wound healing, metastasis, and the microenvironment. Type I collagen coatings on well-plates are used to support adhesion and function of intestinal cells including caco 2 and crypts. Recently developed lyophilized collagen layers and scaffolds have shown superior cellular response and function over PDMS layers, however, these collagen structures have not been incorporated in any microfluidic organotypic device. We describe a novel process to incorporate the membrane into two-chamber microfluidic devices, to show that the devices are biocompatible, and to compare the cell viability with conventional microfluidic devices. We have used caco 2 cells, which have been used to model the human colon in both standard tissue culture plates and microfluidic devices.

Method and Materials

Collagen membrane
10X DMEM (Gibco) was adjusted to pH 7.3 by adding saturated sodium bicarbonate (Sigma). Rat-tail type I collagen solution (BD Biosciences, 3.30 mg/ml) was mixed with the 10X DMEM at a ratio of 9:1, and pH adjusted to 7.2. The collagen solution was spin-coated on a glass slide, frozen at 4, -20, -80 °C for 6 hours separately and placed in a lyophilizer (LABCONCO, Kansas City, MO) overnight. The lyophilized collagen membrane was peeled off and cut to the required shape needed for the microfluidic devices. The fibrous microstructure of the collagen membrane was characterized under a confocal microscope (Carl Zeiss Microscopy, Thornwood, NY).

**Fabrication of the microfluidic device**

We fabricated three types of microfluidic devices with different membranes ([Figure 1D-F](#)) – (1) a single-chamber device with no membrane, (2) a two-chamber device with Transwell polyester membrane (10 um thickness, 0.4 um pore size, Corning, NY), and (3) a two-chamber device with the collagen membrane. The single chamber device was fabricated using standard soft lithography protocols. The two-layer device with the Transwell membrane was fabricated using a method we have developed before. To fabricate the device with the collagen membrane, PDMS monomers (Dow Chemical Co, Midland, MI) were mixed and put in replica mold, vacuumed to remove bubbles inside the PDMS, and put in an oven at 70 °C. The bottom layer was made out of a 250um-thick PDMS sheet that was cut by a laser cutter. Both the PDMS layers were treated in plasma cleaner (Harrick Plasma, Ithaca, NY). The bottom PDMS layer was first bound to a cleaned microscope glass slide. The lyophilized collagen membrane temporarily adhered to the plasma-cleaned top PDMS layer electrostatically. The plasma-cleaned top PDMS layer with the collagen membrane side was inverted, placed on the bottom layer, and then bonded together using the plasma cleaner so that the collagen membrane was securely sandwiched.
between the two PDMS layers. The schematic of the fabrication process is showed in Figures 1A and 1B. Seeded caco 2 cells on the collagen membrane in the device is showed in Figure 1J.

**Mass transport characteristics of the membrane in the device**

Mass transport through the collagen membrane was compared to the Transwell membrane by studying diffusion of FITC dextran (40 kDa molecular weight, Sigma Aldrich, USA) in the two devices. The fluorescence of the transported molecule was measured by a microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA). Devices were assembled, following the protocols described above, each one integrating the Transwell membrane or the collagen membrane. FITC dextran solution (2 mg/mL in DI water) was filled in the upper chamber of the devices, while the lower channels were only filled with DI water. Devices were protected from light throughout the experiment. The liquid from the lower channels was collected after 1, 2, 3, 6, 15, 20, 22, and 28 hours. The collected samples were transferred to a 96-well plate and the fluorescence was read by the microplate reader. Three devices were used for each time point. A standard curve relating the fluorescence of FITC-dextran versus concentration was also constructed. As previous work shows, the linear standard curve of dextran concentration and fluorescence is valid at a low dextran concentration. Therefore, we ranged the concentration of FITC-dextran to 0.01, 0.001, and 0.0001 mg/ml and plotted the standard curve (Figure 2B). The concentration of samples was read from the standard curve (Figure 2C).

We modeled the device as a closed system; in other words, the top and the bottom chambers were taken together as the system since there were no losses from the device during the experiment (Figure 2A). In this case, the total concentration of FITC-dextran in the system was $C_0$, or 2 mg/ml. $C_t(t)$ was the concentration in the top chamber and $C_b(t)$ was the concentration in the bottom chamber. Then,
\[ C_0 = C_t(t) + C_b(t). \]  

(1)

The boundary conditions and initial conditions correspond to

\[ C(x \geq 0, t = 0) = 0, \]  

(2)

\[ C(x \to \infty, t \geq 0) = 0, \]  

(3)

and \[ C(x = 0, t \geq 0) = C_0 - C_b(t). \]  

(4)

To obtain the diffusion coefficient of dextran through membranes, we used the one-dimensional (1D) Fick’s law of diffusion (Eq. 5).

\[
\frac{\partial C(x,t)}{\partial t} = D \frac{\partial^2 C(x,t)}{\partial x^2}
\]

(5)

where \( C(x, t) \) is the concentration of diffusing particles (dextran) as a function of time \( (t) \) and depth \( (x) \), and \( D \) is the diffusion coefficient of dextran through the membranes.

Eq. 5 was solved with these conditions and the solution obtained as Eq. 6

\[
C(x, t) = C \frac{erf_{c} \frac{x}{\sqrt{2Dt}}}{0 \ 1 + erf_{c} \frac{x}{\sqrt{2Dt}}} \]

(6)

where \( erf \) is the error function complement. We adapted the depth \( x \) in Eq. 2 to the thickness \( l \) of membrane\(^{65}\) which modified Equation 6 to

\[
C_l(t) = C_0 \frac{erf_{c} \frac{l}{\sqrt{2Dt}}}{1 + erf_{c} \frac{l}{\sqrt{2Dt}}} \]

(7)

where \( l \) is the thickness of the membrane and \( C_l(t) \) is the dextran concentration at \( x = l \), which is approximately 15 um for collagen membrane and 10 um for Transwell membrane. The model is appropriate because we are interested in determining the transport across the membrane, in other words, at a fixed \( x = l \). Eq. 7 was fitted to the average concentration data as a nonlinear least-square fit by Matlab (The Mathworks, Natick, MA). The curve-fitted plot is shown in Figure 2C.

Cell culture in microfluidic device
Caco 2 cells were seeded in the three devices. Before seeding cells, devices were sterilized under UV light for 15 min. The glass surface of the one-chamber device and the membrane of two-chamber devices were coated by 50 ug/ml fibronectin for 30 min at 37 °C in the incubator. Then, 100 ug/ml type I collagen solution was used to coat the single-chamber device and the two-chamber device with polyester membrane for 30 minutes at 37 °C in the incubator. Caco 2 cells were then seeded in the devices. The cells were cultured for up to days, and the media was changed daily. The cells’ viability was assessed by using a live/dead staining kit (calcein-AM and ethidium homodimer-1, Thermo Fisher); the live and dead cells were quantified using ImageJ (NIH, Bethesda, MD).

**Results and Discussion**

**Type I collagen membrane**

The fibers of the type I collagen fibers on the membrane in the microfluidic devices were well-organized and formed a dense reticular structure (Figure 1G). As the detailed clustered fibers and pores of membrane show, the membrane is evenly woven (Figure 1H). The clustered and densely knitted collagen fibers provided a higher contact area for cells than membranes coated with collagen. We estimated that the diameter of collagen fibers in the membrane was ~1.2 um, while the diameter of the fiber clusters varied from 1 to 20 um. Figure 1J shows that this collagen membrane can well support caco 2 cells.

**Mass transport characteristics of the membrane in the device**

The mass transport and the model are shown in Figure 2A. By using Matlab to fit the experimental data to the developed Eq. 7, we obtained the diffusion coefficient of dextran through the collagen membrane as 4.191×10⁻⁷ cm²/s and through the Transwell membrane as 2.242×10⁻⁸ cm²/s. We estimated that the average pore diameter of the collagen membrane was
10.2 um. The larger pore size of the collagen membrane compared to the Transwell membrane could explain the higher mass transport. The experimental data can now be used to determine transport of proteins and other molecules through the collagen membrane.

**Live/dead analysis**

The live/dead staining results show the growth and death of caco 2 cells in the three devices (Figure 3 A-E). For the single chamber device with no membrane, caco 2 cells were confluent by day three; however, after that, the viability declined, and on day five, the number of cells was lower than the initial seed. For the device with polymer membrane, caco 2 cells multiplied until day three; however, the number of live cells dropped beyond day three; the rate of decline in the number of live cells was lower in this case than that in the single chamber device. In contrast, the cells in the device with collagen membrane showed a steady increase in the number of live cells with no decline even on day five.

The change in the number of dead cells in the devices differed from the growth of cells. For the device without a membrane, there were fewer dead cells until day two; however, beyond that, there was an increase in the number of dead cells. The device with the Transwell membrane showed a similar pattern. However, in the device with the collagen membrane, the number of dead cells decreased significantly on days four and five.

The morphology of caco 2 cells growing on glass and the membranes was quite different between the three devices (Figure 4). Most of caco 2 cells seeded on glass in the single chamber device with no membrane kept the original rounded shape, and even when they were confluent, the adjacent cells did not change their shape (Figures 4A, 4D, and 4G). Cells seeded on the Transwell membrane initially had a rounded morphology (Figure 4B); however, when the cells started to become confluent, the morphology became elliptical (Figures 4E and 4H). On the
collagen membrane device, there are three different morphologies—round, squamous, and collagen fibers integrated (Figure 4J, 4K, and 4L). In the first couple of days, the cells that were not in contact with another cell maintained a round morphology whereas those in contact with the other cells took a squamous appearance (Figure 4C). As the cells became confluent on subsequent days, more cells acquired a squamous appearance, and the cells along the collagen fibers appeared to integrate with the collagen fibers. In fact, on day five, the cells grew into more epithelium and appeared to remodel the microenvironment51 (Figure 4F and 4I). Live/dead staining results (Figure 3) indicated that while the cells on the glass and the polymer membrane start to become apoptotic, the cells on the collagen membrane remained viable.

**Cell viability**

The viability of cells seeded in the three types of devices is shown in Figure 5. Viability of cells in the device with no membrane decreased steadily after day one, with the day five viability being 76%. For the device with the Transwell membrane, although the cells started with a high viability, the viability decreased steadily subsequently with a day five viability of 85%. The viability of the caco 2 cells on the collagen membrane declined initially but increased to >95% on day five (Figure 5). Although during the first couple of days, the viability is similar among the three types of devices (viability > 95%), the advantage of the device with ECM membrane is evident after 48 hours of culture. The higher viability in the ECM device over the other two devices is statistically significant (*). After the third day, the cell viability of the devices without membrane and with polymer membrane continue decreasing while the device with ECM membrane remains at around 95%. This result indicates that the device with ECM membrane supports growth and viability of the cells over the other two devices.

**Conclusions**
We report on the development of a novel integration of a type I collagen membrane into a microfluidic device. The advantages of our approach are that (1) it uses a simple fabrication process that can be easily integrated with multilayer microfluidic devices and (2) the devices are closer physiologically in mimicking the microenvironment than those with a polymer (Transwell) membrane. We characterized the membrane and quantified the mass transport of a high molecular weight fluorescent molecule, which can now be used to predict the transport of proteins through the membrane. We show that cells cultured on the collagen membrane have higher viability than those in a device with no membrane and in one with a Transwell membrane. In addition, integration of the cells with the collagen fibers was an interesting observation, which indicated that the collagen membrane provided a more physiological microenvironment to the cells. The microfluidic device with the collagen membrane can be used to study cell-cell interactions and drug metabolism. Because of the wide use of type I collagen in cell culture and the growing applications of microfluidic-based microphysiological systems, the device with the collagen membrane will be widely applicable to many research areas.

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**Conflicts of Interest**

The authors declare no conflict of interest.

**References**


List of Figures

Figure 1: (A–C) Fabrication of microfluidic devices and lateral views of the devices with cells; (D–F) Microfluidic device with no membrane, sandwiched Transwell membrane, and sandwiched collagen membrane, respectively; (G) Microfluidic device with sandwiched collagen membrane (scale bar, 1000 um); (H) Collagen membrane fiber structure by confocal microscope (scale bar, 100 um); (I) Detailed clustered fibers and pores of the collagen membrane (scale bar, 20 um); (J) Caco 2 cells seeded on lyophilized collagen membrane in microfluidic devices (scale bar, 20 um).

Figure 2: (A) Model scheme of microfluidic device mass transportation; (B) Standard curve of the linear relation of dextran concentration and fluorescence; (C) Dextran concentration at bottom chamber versus time (n=3); circles are the bottom chamber dextran concentration of microfluidic device with collagen membrane; triangles are the bottom chamber dextran concentration of microfluidic device with Transwell membrane.

Figure 3: (A–E) Live and dead staining (calcein-AM and ethidium homodimer-1) of caco 2 cells seeded in microfluidic devices without and with polymer or collagen membranes for five days. (Scale bars, 100 um)

Figure 4: (A–I) The intracellular structure of caco 2 cells growing without and with polymer or collagen membranes for one, three, and five days. Cells were stained by calcein-AM; (J–L) Caco 2 cells seeded on collagen membrane show round, squamous, and collagen fibers integrated appearance, respectively. (Scale bar, 100 um)
Figure 5: Viability of caco 2 cells in three types of devices (without, with polymer, and with collagen membranes) for five days (n > 6); * p value < 0.01.
Figure 1
Figure 3

A: Closed system with Glass slide, Bottom outlet, Top outlet, Top inlet, Bottom inlet, FITC-dextran loaded, Porous membrane, FITC-dextran diffusion.

B: Graph showing fluorescence versus concentration [mg/ml].

C: Graph showing dextran concentration [mg/ml] versus time [h] with Collagen membrane and Transwell membrane.
Figure 4
Figure 5

No membrane | Polymer membrane | Collagen membrane

Day 1

Day 3

Day 5
Figure 6

The graph shows the viability of different treatments over five days (D1 to D5). The treatments include No- (empty square), Polymer (crossed square), and Collagen (filled square). Significance levels are indicated by asterisks: * for P < 0.05 and ** for P < 0.01.
ORGAN-ON-A-CHIP INDUSTRY PROJECT
OBJECTIVE AND SCOPE

• To understand organ-on-chip industry size, market, and growth opportunities

• To conduct a secondary research on the industry in the United States

• To participate in various startup pitch competitions to assess the scope of the idea and seek funding opportunities
INDUSTRY ANALYSIS

• The global organ-on-chip market is poised to grow at a CAGR of around 69.4% over the next decade to reach approximately $6.13 billion by 2025.

• Based on application, the market is categorized into research, agriculture, chemicals, cosmetics, pharmaceuticals, agrochemicals, consumer goods, nutraceuticals, and food and beverages.

• Depending on the organ, the market is segmented by heart-on-chip, human-on-chip, intestine-on-chip, kidney-on-chip, liver-on-chip, and lung-on-chip.

Source: PR Newswire
01
On an average, it takes around 12 years for a drug to travel from a research lab to a patient.

02
Only 5 in 5000 or 10% of the drugs in preclinical testing ever reach clinical trial stages.

03
Only 1 in 5 of these is approved for human use.

04
It costs millions of dollars for a company to develop a new drug.

NEED-GAP
DRUG DISCOVERY – MARKET OPPORTUNITIES

This could perhaps abolish the need for animals in drug development and testing.

HYPE CYCLE FOR EMERGING TECHNOLOGIES

Organ on a chip has created high expectations

Gartner, Inc. Stamford, CT 06902 USA
POTENTIAL MARKET

• The potential markets for this product are the pharmaceutical and biotechnology industries.

• The pharmaceutical/biotechnology global expenditure on R&D for the year 2016 was around $127 billion.

• It is forecasted to reach up to $148 billion by the year 2020.

Source: Pharmasource
TOP COMPANIES

Multinational companies have raised million-dollar investments.

Source: Yole Developpment
KEY TAKEAWAYS

Organ-on-chip is an emerging yet flourishing industry with a market forecast of $6.3bn by 2025.

North America is expected to dominate the industry globally through the forecast period.

Companies are investing Millions of dollars in the Organ on Chip industry.

Potential markets: Pharmaceutical & biotechnology industries spend billions of dollars in drug development.
No salary is requested for Bhushan. Partial salary support is requested for Mehta. Support is requested for a postdoctoral scholar and a graduate student. Materials and supplies are requested to cover the proposed experiments. Partial tuition support is requested for the graduate student.