Effect of Silk-Based Hydrogel Topography on Intestinal Epithelial Cell Morphology and Wound Healing In Vitro

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Effect of silk-based hydrogel topography on intestinal epithelial cell morphology and wound healing \textit{in vitro}

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15 May 2018
Abstract

Recent advances in the field of biomaterials have suggested that cells cultured on substrates resembling the native tissue mechanical properties, matrix and growth factor composition, and topography can adopt phenotypes that more closely resemble the in vivo tissue compared to cells cultured on non-mimetic constructs. Understanding the effect of culture substrate on in vitro tissue formation is important for bioengineering applications that include mechanistic studies of healthy tissue function and development of disease models. In this work, Caco-2 adenocarcinoma cells were seeded on flat and crypt-like topographies of 3D-printed cytocompatible hydrogels derived from silk fibroin protein. Silk hydrogels were selected for their tunable mechanical properties, their ability to support intestinal cell culture, and their relatively slow degradation profile, which was hypothesized to enable the maintenance of substrate topography during culture. 3D-printed acellular silk hydrogels were found to maintain their shape for over eight weeks in culture medium. Cells were seeded on the silk hydrogel substrates, where it was found that cells both attach and proliferate on substrates with flat and intestinal crypt-like topographies. The effect of hydrogel topography on cell morphology and tight junction integrity was assessed using microscopy with immunostaining. In addition, the effect of topography on the closure of disrupted epithelium in both healthy and inflammatory states was assessed in vitro using a wound scratch assay. It is anticipated that the topographical studies presented will assist in developing more physiologically relevant tissue models that may be used to investigate wound healing in the colon.
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>B. mori</td>
<td><em>Bombyx mori</em></td>
</tr>
<tr>
<td>hMSC</td>
<td>Human mesenchymal stem cells</td>
</tr>
<tr>
<td>IESC</td>
<td>Intestinal epithelial stem cells</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>Sodium carbonate</td>
</tr>
<tr>
<td>LiBr</td>
<td>Lithium bromide</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>α-MEM</td>
<td>Minimum Essential Medium Eagle Alpha</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium Eagle</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate-buffered saline</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture polystyrene</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>Vis</td>
<td>Visible light</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly significant difference</td>
</tr>
<tr>
<td>TEER</td>
<td>Transepithelial electrical resistance</td>
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</tbody>
</table>
1. Introduction

Recent advances in the field of biomaterials have demonstrated that cell culture microenvironment, including substrate chemical composition, mechanical properties, and topography, affect cell differentiation and phenotype. In particular, substrate elasticity can direct cell lineage specialization, and culture substrate topography, which affects how cells sense stiffness, can guide cell orientation along constructs, resulting in a specific tissue morphology (Figure 1). Understanding the effect of culture substrate on tissue formation can allow for applications in bioengineering, as tissue remodeling and in vitro disease modeling require scaffolds that regulate proliferation, viability, and intercellular signaling and support extracellular matrix (ECM) deposition.

Hydrogels are three-dimensional polymeric networks comprising cross-linked hydrophilic chains and characterized by high water content and diverse, tunable physical properties. A variety of natural and synthetic polymer hydrogels have been investigated for applications in tissue engineering, drug delivery, and disease modeling. While natural materials, such as alginate and hyaluronic acid, are biocompatible and, in the case of hyaluronic acid, replicate ECM signaling to cells, they do have batch to batch variability and often dissolve or degrade rapidly, making them prone to shape changes. Synthetic materials permit tuning for enhanced control of mechanical properties and degradation, but they cannot signal to cells without adding cell-recognition sequences.

Silk fibroin, a protein derived from Bombyx mori (silkworm) silk cocoons, demonstrates several physical and chemical properties that have given it attention as a biomaterial capable of overcoming the shortcomings of existing hydrogels. Biocompatibility of the silk fibroin is attained by extracting the core silk fibroin protein fibers from the native B. mori silk to remove water-soluble sericin glycoproteins that have been shown to elicit immune responses in vivo.
also possesses high tensile strength and toughness, slow rate of degradation, ease of chemical modification, and the capacity to form a variety of material forms (Figure 2). Silk can be modified to form hydrogels, and highly elastic silk hydrogels with a tunable modulus of elasticity have been attained by reacting silk fibroin solution in an enzyme-catalyzed reaction to induce crosslinking between tyrosine residues. These hydrogels have been shown to support the survival of human mesenchymal stem cells (hMSCs) added to the hydrogel surface and encapsulated in the gel.

The goal of this work is to tune the topography of these cytocompatible silk hydrogels to support intestinal epithelial cell attachment and development into a functional epithelium in vitro. Intestinal epithelium consists of simple columnar cells, or enterocytes, arranged in a crypt structure of glandular microscopic invaginations. The cell layer also includes specialized goblet cells, which secrete mucus to exclude bacteria from the epithelial layer, and intestinal epithelial stem cells (IESCs), which reside at the base of the crypts and control renewal of the epithelial cell layer (Figure 3A). Between these cell types, associated tight and adherens junctions, which consist of a complex of claudins, occludins, junctional adhesion molecules (JAMs), and E-cadherins, effectively seal the paracellular space against passive solute flux (Figure 3B). The cell-to-cell junctions therefore create a selectively permeable epithelial barrier that regulates uptake of nutrients, ions, and solutes, while preventing passage of toxic substances, such as bacteria, bacterial toxins and by-products, and digestive enzymes, from the lumen. The large intestinal epithelium, including the cells and associated junctions, therefore provides both a physical and biochemical barrier that maintains intestinal homeostasis. Due to the importance of the epithelial barrier function, when injury to the epithelium occurs, the cells undergo epithelial restitution, whereby the columnar cells become flattened and migrate into the wound to rapidly restore this barrier, followed by proliferation and differentiation.
Crohn’s disease, a chronic inflammatory bowel disease (IBD), is characterized by severe damage to the mucus layer and mucosal tissue. In particular, there is a decrease in goblet cell number, as well as a disruption of tight junctions that manifests as decreased expression of claudins, occludins, and JAMs (Figure 4). Disruption of the tight junction barrier leads to increased intestinal permeability, which allows penetration of normally excluded luminal antigens, such as bacteria, into the underlying intestinal tissue. These foreign antigens trigger an inflammatory response that leads to increased production and activation of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), which further promote inflammation. TNF-α and other proinflammatory cytokines produced during the inflammatory response have also been shown to further disrupt the tight junction barrier and thus increase permeability. The elevated levels of TNF-α present under Crohn’s disease conditions therefore exacerbate an inflammatory response by allowing increased permeation of antigens. As a result of this inflammatory state, altered wound healing also becomes a characteristic of the Crohn’s disease phenotype. To be able to develop treatments for intestinal lesions present in Crohn’s disease, the

Figure 3: Structure of healthy large intestinal epithelium. (A) The epithelium consists of a monolayer of mature enterocytes, mucus-producing goblet cells, and intestinal epithelial stem cells (IESCs) (Adapted from Peterson, L.W.; Artis, D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nature Reviews Immunology* 2014, 14, 141-153). (B) Intestinal epithelial cells are associated with tight junction molecules (claudins, occludins, and JAMs) and adherens junction molecules (E-cadherins) that form the epithelial barrier (Hammer, A.M.; Morris, N.L.; Earley, Z.M.; Choudhry, M.A. The first line of defense: The effects of alcohol on post-burn intestinal barrier, immune cells, and microbiome. *The Journal of the National Institute on Alcohol Abuse and Alcoholism* 2015, 37 (2), 209-202).
physiology and pathophysiology of the intestinal epithelium and intestinal epithelial cell wound healing must be further investigated.\textsuperscript{19}

When modeling intestinal epithelium \textit{in vitro}, it is difficult to expand human intestinal epithelial cells into a functional cell layer. Over recent decades, the Caco-2 cell line, derived from a human colon adenocarcinoma, has been used to model intestinal epithelium.\textsuperscript{22, 23} After reaching confluence, the cell line has been shown to spontaneously differentiate into a polarized monolayer of cells possessing the morphology, cell junctions, and functional characteristics of intestinal enterocytes in native epithelium within three weeks.\textsuperscript{22, 23} To assess whether a functional intestinal epithelial layer has formed in the laboratory, morphology, polarity, and cell layer permeability are typically assessed. Visualization of morphology can be performed using confocal and electron microscopy, which will show a confluent cell monolayer containing an apical brush border.\textsuperscript{23, 24} Differentiation into a polarized layer can also be quantified by measuring the activity of brush border enzymes such as alkaline phosphatase.\textsuperscript{23, 24} Assessment of the cell layer’s junction integrity and barrier function is achieved by its monitoring permeability and resistance to paracellular...
transport with transepithelial electrical resistance (TEER).\textsuperscript{23, 24}

To simulate \textit{in vitro} the diseased state of the large intestinal epithelium, inflammatory cues, such as the inflammatory TNF-\(\alpha\), will be used to alter Caco-2 cell morphology and function. In the Crohn’s disease phenotype \textit{in vivo}, TNF-\(\alpha\) activates components of the NF-\(\kappa\)B pathway to promote a pro-inflammatory state\textsuperscript{25} while also disrupting the tight junction barrier and increasing intestinal epithelial permeability.\textsuperscript{17} \textit{In vitro}, TNF-\(\alpha\) has been shown to have the same effect.\textsuperscript{17} Treatment of a cell monolayer with TNF-\(\alpha\) at a concentration of 10 ng/mL for 48 hours has been shown to disrupt the intestinal epithelial tight junction barrier by modifying the expression level and localization of tight junction proteins.\textsuperscript{17, 26, 27} It has been proposed that TNF-\(\alpha\) induces such tight junction remodeling through activation of the NF-\(\kappa\)B molecular pathway, which ultimately leads to “tight” tight junction proteins being replaced with “leaky” tight junction proteins (Figure 5).\textsuperscript{26} According to this proposed method, TNF-\(\alpha\) causes disruption in the tight junction barrier by affecting tight junction protein expression and inducing cycling of the proteins into the cell. This method supports findings that TNF-\(\alpha\) causes decreased expression and continuity of zonula occludens-1 protein,\textsuperscript{17, 28} increased cellular uptake of sealing claudin proteins, such as claudin-1 and claudin-5,\textsuperscript{26, 28, 29} and upregulated expression of pore-forming claudin proteins, such as claudin-2.\textsuperscript{27} By reducing the presence of tight junction proteins that seal the intestinal epithelial barrier and replacing them with leaky tight junction proteins, TNF-\(\alpha\) disrupts the epithelial barrier of the large intestine. Since TNF-\(\alpha\) induces tight junction disruption that is seen in the Crohn’s disease phenotype \textit{in vivo}, it is anticipated that TNF-\(\alpha\) treatment of a Caco-2 cell monolayer will be sufficient to simulate the diseased state \textit{in vitro}.

\textit{In vitro} modeling can be used to gain an increased understanding of both the physiology and pathophysiology of the large intestinal epithelium.\textsuperscript{30} This enhanced understanding of both normal and aberrant functioning can pave the way for advancements towards pharmacological treatments of Crohn’s disease and other intestinal diseases.\textsuperscript{31, 32} The applications of \textit{in vitro} modeling therefore demand that the models used display physiological relevance to the \textit{in vivo} large intestine.\textsuperscript{32}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure5.png}
\caption{Mechanism of tight junction remodeling by TNF-\(\alpha\) (Capaldo, C. T.; Nusrat, A. Biochimica et Biophysica Acta (BBA) - Biomembranes \textbf{2009}, \textit{1788} (4), 864–871.)}
\end{figure}
Traditional methods of two-dimensional cell culture with Caco-2 cells lack this capability, as a monolayer of enterocytes on a flat surface cannot recapitulate the complexity of the in vivo cell layer and environment. Interest has therefore shifted towards developing three-dimensional models, whose topography and composition can be tuned to simulate the in vivo cell microenvironment.

In this study, it was hypothesized that Caco-2 cells cultured on silk hydrogels with crypt-like topographies will display cell and tight junction morphologies that more closely resemble those observed in vivo compared to cells cultured on flat topographies, and that these changes may alter epithelial cell movement in response to a wound scratch in vitro. Furthermore, cells cultured in the presence of inflammatory cues will demonstrate impaired wound closure and decreased junction integrity compared to those cultured in the absence of these factors. It is expected that this tunable hydrogel model can serve as a baseline upon which future modifications, such as introduction of different cells types, can be made, in order to capture the complexity of the large intestinal epithelium.
2. Materials and Methods:

2.1. Materials & Reagents

2.1.1. Silk Fibroin Extraction

*Bombyx mori* silk cocoons were purchased as HI-Silk 21 from Kawaguchi Shoten Corporation, Ltd (Nagoya, Japan). Sodium carbonate and lithium bromide were purchased from Sigma-Aldrich (USA). Fisherbrand™ regenerated cellulose dialysis tubing (MWCO 3,500) was purchased from ThermoFisher Scientific (USA).

2.1.2. Hydrogel Preparation

For silk-based hydrogel preparation, hydrogen peroxide and horseradish peroxidase (HRP) was purchased from Sigma-Aldrich (USA). For alginate hydrogel preparation, alginic acid with sodium salt from brown algae (sodium alginate) and calcium chloride were purchased from Sigma-Aldrich (USA). For use as a substrate in hydrogel 3D-printing, polydimethylsiloxane (PDMS, Sylgard 184) by DowCorning Co. was purchased from Ellsworth Adhesives (USA).

2.1.4. Caco-2 Cell Culture

The Caco-2 cell line (ATCC® HTB-37™) was obtained from American Type Culture Collection® (ATCC, USA). Caco-2 cell culture medium, including Minimum Essential Medium Eagle Alpha (α-MEM) and Minimum Essential Medium Eagle (MEM), was purchased from ThermoFisher Scientific (USA). Trypsin, fetal bovine serum (FBS), penicillin, streptomycin, fungicide, non-essential amino acids, and sodium pyruvate, as well as 1X Dulbecco’s phosphate-buffered saline (DPBS), were also purchased from ThermoFisher Scientific (USA). Human Recombinant Tumor Necrosis Factor-Alpha (TNF-α) was purchased from PeproTech (USA). Gelatin from porcine skin was purchased from Sigma-Aldrich, and fibronectin from bovine plasma were purchased from ThermoFisher Scientific (USA).

For sterile filtration, Nalgene Rapid-Flow Bottle Top Filters (0.2 μm) and Fisherbrand™ sterile syringe filters (0.22 μm) were purchased from ThermoFisher Scientific (USA).

Corning® Falcon™ 12-well, 24-well, and 48-well tissue culture polystyrene (TCP) cell culture plates were purchased from ThermoFisher Scientific (USA). Corning® T-75 and T-175 cell
culture flasks with surface areas of 75 cm\(^2\) and 175 cm\(^2\), respectively, were purchased from Sigma-Aldrich (USA).

### 2.1.5. Cell Culture Analysis

Pierce\textsuperscript{TM} Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit was purchased from ThermoFisher Scientific (USA), as was Alamar Blue Assay reagent. Hoechst 33342 Fluorescent Stain and Calcein AM dye were purchased from ThermoFisher Scientific (USA). Claudin-1 Antibody (A-9) conjugated to fluorescein isothiocyanate (FITC, 200 μg/mL) was purchased from Santa Cruz Biotechnology, Inc.

For stereomicroscope and optical microscope imaging, FisherBrand\textsuperscript{TM} plastic petri dishes were purchased from ThermoFisher Scientific (USA). Molecular Probes\textsuperscript{®} 96-well microplates for fluorescence-based assays and Corning\textsuperscript{®} clear 96-well clear microplates for absorbance-based assays were purchased from ThermoFisher Scientific (USA). For confocal microscopy imaging, 35-mm coverslip-bottom dishes were purchased from MatTek Co. (USA).

### 2.2. Instrumentation

#### 2.2.2. 3D Printing

AutoCAD\textsuperscript{®} by Autodesk Incorporated was used to design flat and crypt-like topographies of hydrogel. For 3D-printing of hydrogels, the Hyrel system 30M printer was used.

#### 2.2.3. Imaging

For imaging of shape fidelity samples, the Nikon SMZ-10 Binocular Stereo Zoom microscope (stereomicroscope) and AmScope ME 520TA Metallurgical microscope (optical microscope) were used, along with AmScope v3.7 imaging software. For image collection in wound healing studies, the Zeiss Axiovert 200M microscope was used with Micro-Manager v1.4.22 software. For hydrogel morphology and tight junction imaging, the Leica SP8 Spectral Confocal microscope was used in conjunction with Leica Application Suite X v3.3.0 image acquisition and processing software.

#### 2.2.4. Quantitative Analysis
For image analysis in shape fidelity studies, ImageJ v1.51k (Wayne Rasband, National Institutes of Health, USA) was downloaded from the National Institutes of Health website (https://imagej.nih.gov/ij/download.html). For image analysis of wound scratch assays, TScratch v1.0 was downloaded from the Computational Science & Engineering Laboratory website (https://github.com/cselab/TScratch).

For analysis of ultraviolet/visible light (UV/Vis) applications, the BioTek™ Synergy™ HT Multi-Detection Microplate Reader and the associated BioTek™ Gen5™ data analysis software were used.

2.2.5. Statistical Analysis

GraphPad Software (2018), which employs the Grubbs’ test, was used to perform outlier detection. OriginPro v8.5 by OriginLab was used for statistical analysis.

2.3. Methodology

2.3.1. Silk Fibroin Extraction

The protocol for extraction and preparation of purified silk fibroin solution from the B. mori silk cocoon followed the published literature. Briefly, cut silk cocoons were boiled in 0.02M aqueous Na₂CO₃ for 30 minutes, then fibers were rinsed three times for 20 minutes in deionized water (1 L per batch), dried at room temperature overnight, and dissolved in 9.3M LiBr at 60°C for 4 hours. The resulting silk fibroin solution was then dialyzed in dialysis tubing (MWCO 3,500) against ultrapure water for 48 hours, centrifuged twice at 12,700 x g for 20 min, and stored at 4°C.

2.3.2. Silk-Based Hydrogel Preparation

According to published protocol for silk fibroin hydrogel synthesis, 10 μL of sterile-filtered (0.22 μm) 165 mM H₂O₂ and 10 μL of sterile-filtered (0.22 μm) 1000 U/mL HRP solution was added to 1 mL of sterile-filtered (0.22 μm) 2-4% (w/v) silk solution. The reacting solution crosslinked at room temperature to form a gel at a concentration-dependent rate. To cast silk hydrogels with a flat topography (hereafter “casted flat hydrogels”), the reaction mixture was added to a 12-well plate and allowed to cure for two hours at 37°C in a cell culture incubator with a humidified atmosphere containing 5% CO₂. When 3D-printing the flat and crypt-like
topographies of hydrogel (hereafter “flat hydrogels” and “crypt-like hydrogels,” respectively), a two-stage crosslinking method was used. In this method, a partially crosslinked solution was first prepared by adding a concentration-dependent amount of 165 mM H₂O₂. Since more concentrated silk fibroin solutions require a lower volume of H₂O₂ to induce partial crosslinking, H₂O₂ was added in values ranging from 1.3-1.4 µL per mL for 2% (w/v) silk fibroin solution to 1.9-2.2 µL per mL for 4% (w/v) solution (Table 1). This mixture was then 3D-printed using robotic dispensing, whereby the reacting solution that forms the gel is extruded through a syringe with a 27G (200 µm inner diameter) needle onto PDMS substrate. Robotic dispensing was performed using a volumetric flow rate of 0.0011 mL/s and a printing speed of 10 mm/s. PDMS substrate was used due to its hydrophobic nature, which prevents the ink from flattening out and allows the gel to hold its shape during printing, and its sticky consistency, which aids in deposition of the solution. The different topographies, including flat topography gels and crypt-like topography gels with microscopic (1000-µm diameter, 500-µm depth) holes, were initially designed in auto-CAD and ultimately exported to a G-code that is read by the printer. Although the native intestinal epithelium contains crypts with diameters of 50-100 µm and depths of 300-400 µm, the crypt-like topography was designed with larger dimensions to account for gel shrinking due to printing and processing. After printing, 50 µL of dilute H₂O₂ solution is added to the hydrogels. Since silk hydrogels of higher silk fibroin concentration require a lower concentration of H₂O₂ to complete crosslinking, H₂O₂ is added at concentrations ranging from 2.38 mM for 1% (w/v) silk to 2.16 mM for 4% (w/v) silk (Table 1). After 1 hour, the constructs were blotted with a Kimwipe™ and stored at room temperature for 2-3 weeks in asterile deionized water.

To ensure sterile conditions for all studies, casted silk hydrogels in a 12-well plate were incubated in 3 mL of 70% ethanol for 1-2 hours, and flat and crypt-like silk hydrogels were placed in a 24-well plate and incubated in 2.5 mL of 70% ethanol for 1-2 hours. After this incubation, the ethanol was replaced for overnight incubation at 37°C in a humidified atmosphere of 5% carbon
dioxide. The next day, the 3D-printed gels were steriley transferred to a new 24-well plate. After five 3-minute washes with 2.5 mL of sterile water, the sterile water was replaced every hour for 5-6 hours to remove residual ethanol and result in a sterilized construct.

2.3.3. Alginate Hydrogel Preparation

A 3% (w/v) alginate solution was prepared by dissolving sodium alginate (source) in deionized water and stirring at room temperature overnight. After dissolution, 60 mM CaCl₂ was added to induce crosslinking, resulting in a final solution concentration of 2.65% (w/v) alginate. Although literature indicated that 2% (w/v) alginate hydrogels could successfully be 3D-printed, the concentration of alginate used for 3D printing was ultimately increased to 2.65% due to experienced difficulties in printing. To ensure an even crosslinking density, the solution was mixed and vortexed thoroughly. To 3D-print alginate crypt-like hydrogels with microscopic (1000-μm diameter, 500-μm depth) holes, the topography was designed in auto-CAD, exported by a G-code, and printed onto PDMS substrate using robotic dispensing. After printing, 50 μL of 0.1M CaCl₂ was added to the gels and allowed to incubate for 10 minutes in order to complete the crosslinking process. The hydrogel was then removed from the PDMS substrate and stored at room temperature in 0.1M CaCl₂.

To ensure sterile conditions, alginate gels were placed in a 24-well plate and incubated in 2.5 mL of 70% ethanol supplemented with 0.1M CaCl₂ for 1-2 hours, at which time the ethanol with CaCl₂ was replaced for overnight incubation at 37°C in a humidified atmosphere of 5% CO₂. The next day, gels were steriley transferred to a new 24-well plate. After five 3-minute washes with 2.5 mL of sterile water with 0.1M CaCl₂, the water was replaced every hour for 5-6 hours. Sterile alginate hydrogels were stored at room temperature in 0.1M CaCl₂ for 6 days, at which time a shape fidelity study was begun.

2.3.4. Silk-Based Hydrogel Shape Fidelity Study

After preparing and sterilizing 3D-printed flat ($n = 3$) and crypt-like ($n = 3$) 3% (w/v) silk-based hydrogels and transferring them to 24-well plates, the working volume (500 μL) of α-MEM media was added to the gels 18 hours before beginning the shape fidelity study to allow for equilibration. Flat and crypt-like silk hydrogels in 500 μL α-MEM media were imaged steriley with the stereomicroscope and optical microscope at 1.6X and 5X, respectively, at time points over
62 days. Crypt-like hydrogels were imaged in four quadrants to allow the entire scaffold to be imaged. α-MEM was replaced every 3-4 days to maintain constant media levels. For flat gels, the side lengths of each hydrogel at every time point was measured using ImageJ, and percent change in side length compared to Day 0 was calculated. For crypt-like gels, the hole diameters in each hydrogel at every time point were measured using ImageJ.

2.3.5. Alginate Hydrogel Shape Fidelity Study

Crypt-like \((n = 4)\) alginate hydrogels in 500 μL MEM media supplemented with 22.5 mM CaCl₂ were imaged steriley with the optical microscope at 5X at time points over 15 days. MEM with CaCl₂ was replaced every 2-3 days to maintain constant media levels. To assess changes in shape fidelity, ease of hydrogel handling was noted, and the hole diameters in each hydrogel at every time point were measured using ImageJ.

2.3.6. Caco-2 Cell Culture

Caco-2 cells were cultured in media consisting of MEM containing 10% FBS, 1% penicillin/streptomycin/fungicide, 1% non-essential amino acids, and 1% sodium pyruvate and incubated at 37°C in a humidified atmosphere of 5% CO₂. Media was changed every 2-3 days, and cells were passaged at 50% confluence during expansion.

2.3.7. Wound Healing on TCP: Method Development & TNF-α Screening

As illustrated in Figure 6, Caco-2 cells were seeded at 30,700 cells/cm² in a working volume of 1 mL on 12-well TCP plates. After 7 days, 48 hours before the wound scratch, media was changed from complete media to complete media containing 0 ng/mL \((n = 3)\), 5 ng/mL \((n = 3)\), 10 ng/mL \((n = 3)\), or 25 ng/mL \((n = 3)\) TNF-α. Per published wound scratch assay protocols in

Figure 6: Protocol for conducting wound healing studies with Caco-2 cells on TCP (Adapated from Stamm, A.; Reimers, K.; Strauβ, S.; Vogt, P.; Scheper, T.; Pepelanova, I. BioNanoMaterials 2017, 17 (1-2), 79-87).
24 hours before the wound scratch, all media was changed to serum-deprived media containing 0.1% FBS with the appropriate amount of TNF-α in order to limit wound closure due to cell proliferation. On the day of the wound scratch, 750 μL cell culture supernatant was removed from each well to Eppendorf tubes. The supernatant was then placed in -20°C to allow the degree of cell death from TNF-α treatment to be assessed by an LDH assay. Remaining media was aspirated, and cell layers were washed once with 1 mL of 1X DPBS. To induce the wound scratch, a 200-μL pipette tip was used to scratch each surface in a “t” shape. Cells were washed again with 1 mL of 1X DPBS before replenishing samples with the appropriate media. Wounds were imaged using the Zeiss AxioVert 200M (10X, phase-contrast, transmitted light) at 0, 2, 4, 6, 8, and 27 hours, and the percent of wound closure compared to time t = 0 was assessed using TScratch software.

To quantify cell death that took place during TNF-α treatment, an LDH assay (Figure 7) was performed to determine the LDH activity of cell culture supernatant from the wound healing study on TCP, per the protocol of the LDH Cytotoxicity Assay Kit. In damaged cells, cell lysis causes LDH to be released from the cytoplasm. LDH facilitates an oxidation-reduction reaction whereby lactate is oxidized to pyruvate and NAD+ is reduced to NADH. The NADH can then react to produce formazan, a colored product whose absorbance can be measured at 490 nm. A large degree of cell death would therefore result in higher absorbance values being recorded.

![Diagram of LDH cytotoxicity assay mechanism](image)

**Figure 7:** Schematic of LDH cytotoxicity assay mechanism (Adapted from Pierce LDH Cytotoxicity Assay Kit. *Thermo Scientific 2014*).

### 2.3.8. Caco-2 Cell Culture on Casted Hydrogels

After preparing and sterilizing casted silk hydrogels in 12-well plates, the working volume (1
mL) of complete media is added to the gels 48 hours before cell seeding to allow for equilibration of the construct. On the day of cell seeding, sterile-filtered (0.22 μm) gelatin (0.2 mg/mL), which increases cell attachment to hydrogels, is added at the volume required to coat the gels (500 μL) and allowed to sit for three hours at 37°C in a humidified atmosphere of 5% CO₂. Gelatin is then aspirated and 500 μL of concentrated cell suspension (61,400 cells/mL) is added to the gels to achieve a final seeding density of 30,700 cells/cm². After three more hours of incubation at 37°C, the volume of complete media necessary to reach the working volume of 1 mL is added to the gels.

2.3.9. Caco-2 Cell Culture on 3D-Printed Hydrogels

The experimental setup for Caco-2 cell culture on 3D-printed flat and crypt like hydrogels is shown in Figure 8. To ensure cell attachment to hydrogels rather than to the TCP well, a volume of 9 μL of PDMS mixture was added to coat the bottom of each well of a 48-well plate. The PDMS coating was then sterilized with a 30-minute UV treatment and allowed to cure at room temperature for 2 days. After curing, the PDMS was further sterilized with a 4-hour incubation in 70% ethanol and then, after aspirating the ethanol, another 30-minute UV treatment. To allow for gel equilibration, the working volume (500 μL) of complete media was added to the sterile flat and crypt-like hydrogels 48 hours before cell seeding. To promote cell attachment, proliferation, and migration, the day before cell seeding, a sterile filtered solution of gelatin (0.2 mg/mL) and fibronectin (0.004 mg/mL) was added to each hydrogel at the volume required to coat the flat and crypt-like gels. The gelatin/fibronectin solution was then aspirated, and the coated scaffolds were transferred onto the sterile PDMS. Sterile autoclave tubing with an outer diameter of approximately 11.1 mm was cut to have a 9.5 mm outer diameter, 4.77 mm inner diameter, and 3

![Figure 8: Protocol for Caco-2 cell culture on hydrogels.](image)
mm thickness. The tubing was then securely placed on top of the scaffold. In order to achieve a final seeding density of 300,000 cells/cm² for flat gels and from 281,426 to 321,200 cells/cm² for crypt-like gels, 40 µL of a concentration cell suspension (1.343 x 10⁶ cell/mL and 2.403 x 10⁶ cells/mL for flat and crypt-like gels, respectively) was added. After five hours of incubation at 37°C in a humidified atmosphere of 5% CO₂, the working volume (240 µL) of media was added to each sample.

For all TCP controls performed with studies on gels, cells were seeded onto 48-well plates at 72,000 cells/cm² in a 240-µL cell suspension. Caco-2 cells were seeded at a lower seeding density due to the anticipation of lower cell attachment to flat and crypt-like gel samples.

2.3.10. Cell Attachment and Proliferation on Hydrogels

Alamar Blue assay was performed on Caco-2 cells seeded on flat and crypt-like hydrogels at 300,000 cells/cm² and on TCP at 72,000 cells/cm². The active ingredient of Alamar Blue, resazurin, is a blue, nonfluorescent, cell permeable indicator dye that becomes converted to resorufin, a red-fluorescent compound, via reduction reactions in metabolically active cells (Figure 9). The amount of fluorescence produced from a sample is proportional to the number of living cells. To quantify the cell number, fluorescence at 540 nm excitation and 590 nm emission was recorded at time points over 21 days. After normalizing these values by subtraction of acellular

![Figure 9: Schematic of Alamar Blue assay mechanism](Resazurin Cell Viability Assay. Canvax, 2014).
controls, the fluorescence compared to Day 1 was calculated by dividing the normalized fluorescence at each time point by the value on Day 1. Proliferation of Caco-2 cells was thus quantified by an increase in metabolic activity.

To assess the degree of cell attachment onto samples, known concentrations of cell suspension ranging from 1,200 cells/sample to 68,400 cells/sample were added to 48-well plates in a working volume of 240 μL. On Day 1, an Alamar Blue assay was performed on these known samples, and fluorescence at 540 nm excitation and 590 nm emission was related to the number of cells per sample using the calibration curve.

2.3.11. Cell Morphology on Hydrogels

Caco-2 cells seeded on flat (n = 3) and crypt-like (n = 3) silk hydrogels were stained with 500 μL fluorescent dye containing 1% (v/v) Hoechst stain and 0.08% (v/v) Calcein AM to allow for visualization of cell nuclei and cytosol products, respectively. At time points over 21 days, Z-stacks were performed on one flat and one crypt-like hydrogel using confocal microscopy at 10X and 40X magnification.

2.3.14. Tight Junction Integrity on Hydrogels

For immunofluorescence staining of Caco-2 cells on flat (n = 2) and crypt-like (n = 2) silk hydrogels, claudin-1 antibody was diluted 1:50 in 1X DPBS. Forty-five days after cell seeding, cell layers were washed with 1X DPBS before incubation in 500 μL of the staining solution for 30 minutes at 37°C. Hydrogels were imaged at 40X magnification using confocal microscopy.

2.3.12. Wound Healing on Hydrogels

In a preliminary study to assess the previously developed wound scratch method, flat hydrogels prepared from 2% and 4% (w/v) silk fibroin solution were cast in 12-well plates at thicknesses of 150 μm (n = 3, per concentration) and 450 μm (n = 3, per concentration). Cells were seeded at 61,400 cells/cm² from a 500-μL cell suspension and were flooded with 500 μL media after the three-hour incubation. 24 hours before the wound scratch, all media was changed to serum-deprived media containing 0.1% FBS with the appropriate amount of TNF-α to limit wound closure due to cell proliferation. On the day of the wound scratch, media was aspirated, and cell layers were washed once with 1 mL of 1X DPBS. To induce the wound scratch, a 200-μL pipette
tip was used to scratch each surface in a “t” shape. Cells were washed again with 1 mL of 1X DPBS before replenishing samples with the media. Wounds were imaged using the Zeiss AxioVert 200M (10X, phase-contrast, transmitted light) at 0, 2, and 4 hours.

To assess the effect of hydrogel topography on wound healing, the wound scratch method previously developed for Caco-2 cells on TCP was applied to Caco-2 cells grown on flat \((n = 10)\) and crypt-like \((n = 10)\) silk-based hydrogels. As a control, cells were also seeded on TCP \((n = 10)\). After 19 days, 48 hours before the wound scratch, media was changed from complete media to complete media containing 0 ng/mL \((n = 5, \text{per topography})\) or 10 ng/mL \((n = 5, \text{per topography})\), simulating the healthy and diseased states, respectively. 24 hours before the wound scratch, all media was changed to serum-deprived media containing 0.1% FBS with the appropriate amount of TNF-α. To induce the wound scratch, a 200-μL pipette tip was used to scratch each surface in a “t” shape. Wounds were imaged using the Zeiss AxioVert 200M (10X, phase-contrast, transmitted light) at 0, 3, 6.5, 10, and 24.5 hours, and the percent of wound closure compared to time \(t = 0\) was assessed using TScratch software. The wound healing method used on 3D-printed hydrogels is shown in Figure 10.

2.3.15. Statistical Analysis

The Grubb’s test was used to perform outlier detection using a significance level of 0.05. Origin software was used to analyze statistical significance via one-way analysis of variance (ANOVA) with post-hoc Tukey honestly significant difference \((HSD)\) test. Statistical significance is reported at *\(p < 0.05\); **\(p < 0.005\); ***\(p < 0.001\).
3. Results and Discussion

3.1. Shape Fidelity Study

The shape fidelity of flat and crypt-like silk hydrogels were assessed by imaging the gels at time points over 62 days (Figure 11A) and subsequently measuring the side lengths and hole diameters, respectively, with ImageJ software (Figure A.1.1). For flat hydrogels, horizontal and vertical side lengths were averaged for each sample, and the percent change in side length compared to Day 0 were calculated at each time point (Figure 11B). For crypt-like hydrogels, the

Figure 11: Shape fidelity study conducted with 3D-printed flat (n=3) and crypt-like (n=3) 3% (w/v) silk hydrogels. (A) Representative samples, imaged at 1.6X or 5X magnification for flat and crypt-like gels, respectively, are depicted. (B) Percent change in side length for flat hydrogels compared to the Day 0 measurements, reported for one sample. (C) Average hole diameters for crypt-like hydrogels, reported for one sample. Error bars represent standard deviation. Significant at *p < 0.05; **p < 0.005; ***p < 0.001.
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hydrogel was imaged in four quadrants (top left, top right, bottom left, and bottom right), and the hole diameters of all crypts in a scaffold were averaged at each time point (Figure 11C).

Statistical analysis of the percent change in side length for flat gels indicates significant (p < 0.001) changes in side length compared from Day 0 for all time points. There is an overall decrease in side length of approximately 15% by Day 62. Although the decrease in side length is statistically significant, it should be investigated whether the 15% change is reproducible. If reproducibility is demonstrated, the hydrogel can then be 3D-printed at a larger size that will allow the final designed dimensions to be achieved after the shape change. Furthermore, hydrogels made from other natural biomaterials undergo even more significant and more rapid shape changes and degradation, with collagen hydrogels reported to experience a 60% decrease in size within 5 days in the presence of lung fibroblasts.45 As a result, use of this hydrogel in cell culture applications can still be supported. This conclusion can be further justified through qualitative observations of the gel; although the measured side length may have decreased, the hydrogel was still capable of being handled without tearing throughout the 8-week study, and all artifacts in the gel, including ridges and indentations, are present at each time point. Based on the observed retention of shape fidelity, it seems that fluctuations in the measured hydrogel side length may be due to limitations of the microscope equipment, as the stereomicroscope image is sensitive to sample placement. Since the hydrogels tend to curl and do not lay perfectly flat, the two-dimensional image produced may distort the dimensions of the side length as well.

Analysis of the crypt-like hydrogels indicate that the initial hole size ranges from approximately 290 to 570 μm, and that there is only a significant decrease in hole size at the Day 62 time point. Recalling that the auto-CAD design of the crypt-like gels specifies a hole diameter of 1000 μm, it is evident that printing and post-printing processing results in the holes shrinking. From Day 0 through Day 56, however, the average hole diameters remain between 430 and 350 μm, with no statistically significant changes observed. Of note, the error bars for each time point span a range of 200-300 μm, with the largest standard deviations occurring at time points after Day 28. The wide spread of data can be attributed to inconsistency in hole size throughout a single scaffold. Differing placement of the scaffold could also cause holes to appear more oblong than circular upon imaging. Furthermore, over the course of the 8-week study, it was observed that some holes merged together while others closed, resulting in a large standard deviation but an average hole diameter that was not significantly different from previous time points. Despite these
variations, the crypt-like hydrogels, like the flat hydrogels, retained their ease of handling throughout the study.

Based on the results of this shape fidelity study, it can be concluded that the silk-based hydrogels retain their shape fidelity over 8 weeks. Although there are statistically significant changes at certain time points for certain gel samples, the fluctuations remain less than a 30-35% decrease. As previously mentioned, these changes could be attributed to placement of the scaffold and sensitivity of the microscope equipment. Fluctuations observed could also be attributed to inconsistencies in the time it took to image the gels, as the gels tend to shrink after being out of media. Ultimately, it seems that quantitative fluctuations observed through the 8-week mark can be attributed to external factors rather than to properties of the silk hydrogel material.

Another means of verifying the silk hydrogel shape fidelity was by comparison to alginate hydrogels. Alginate and silk are both natural biomaterials investigated for cell culture applications, but silk has been shown to exhibit slow degradation rates. Using a similar protocol to the silk hydrogel shape fidelity study, alginate hydrogels were imaged in quadrants at time points over two weeks (Figure 12A). The average hole diameter from each scaffold was calculated at each time point (Figure 12B). From Day 0 through Day 5, there was no significant difference in hole diameter. By the Day 9 time point, however, the hydrogel began to tear upon handling. For most hydrogels, the tearing disrupted the crypts, making quantitative measurements of hole size more difficult, but, when the holes remained intact, the diameter decreased. The decrease in shape

![Figure 12: Shape fidelity study conducted with 3D-printed crypt-like (n=4) alginate hydrogels. (A) Representative samples, imaged at 5X magnification, are depicted. (B) Average hole diameters for crypt-like hydrogels, reported for one sample. Error bars represent standard deviation. Significant at *p < 0.05; **p < 0.005; ***p < 0.001.](image)
fidelity by Day 12 is therefore indicated not only by changes in hole size, but also by difficulty in handling, leading to tearing, gel degradation, and, as a result, merging of holes.

The results of the alginate shape fidelity study serve to further enhance the conclusion that silk-based hydrogels retain their long-term shape fidelity. Establishing this shape fidelity was a crucial step that will help to justify the use of these silk hydrogels as a biomaterial with long-term cell culture.

3.2. Wound Healing on TCP: Method Development & TNF-α Screening

The method for conducting a wound healing study with Caco-2 cells on TCP was the product of literature review and preliminary wound healing experimentation. The length of TNF-α treatment and serum starvation, as well as the technique of washing the cell layer before and after inducing the wound scratch, were derived from literature. The duration of Caco-2 cell culture before the wound scratch and the method of both inducing and imaging the wound scratch were determined using trial and error. It is believed that the procedure proposed in the Methods section is an optimized technique for performing wound scratch assays on TCP.

Wound scratches induced in confluent Caco-2 cell layers treated with either 0 ng/mL (Control), 5 ng/mL, 10 ng/mL, or 25 ng/mL of TNF-α for 48 hours and serum-starved for 24 hours were imaged at time points over 27 hours (Figure 13A-D). For quantitative analysis, TScratch software marked the original wound and then calculated the percent of open wound area remaining at each time point. For the 0 ng/mL control samples, 40% of the original wound area remained after 27 hours, on average, indicating an approximate wound closure of 60%. The 10 ng/mL samples, however, only achieved an average of 20% wound closure by the 27-hour time point. TScratch and Origin Analysis indicated that, at the 27 hour time point, there is a statistically significant difference (p<0.05) between the Control (0 ng/mL) and the 10 ng/mL samples (Figure 13E). The results of the wound scratch assay therefore indicate that 10 ng/mL of TNF-α is sufficient to impair wound closure of Caco-2 cells. Since impaired wound closure would be characteristic of the inflammatory, or diseased, state, it seemed that a 48-hour treatment with 10 ng/mL TNF-α is a suitable method of simulating the diseased state in future studies.

To confirm that the impaired wound closure with 10 ng/mL TNF-α is, in fact, due to the inflammatory effects of the cytokine rather than due to increased cell death from TNF-α treatment, an LDH assay was conducted on the cell culture supernatant from each experimental sample. Using
LDH activity as an indication of cell death, the LDH assay results confirm that there is not a
significant degree of cell death from cell treatment with any concentration of TNF-α (Figure 13F). As a result, it can be reasonably concluded from this wound healing study that 10 ng/mL TNF-α is sufficient to simulate the inflammatory state without inducing cell death. This finding is consistent with literature, which suggests that a 48-hour treatment with 10 ng/mL TNF-α is sufficient to induce changes in the Caco-2 cell layer, such as disruption of tight junctions, that are characteristic of inflammatory bowel disease.\textsuperscript{17, 26, 27} As a result, in all subsequent wound healing and morphology studies, this concentration of TNF-α is used to represent the diseased state.

3.3. Cell Attachment and Proliferation on Hydrogels

Upon beginning Caco-2 cell culture on silk hydrogels, it was crucial to demonstrate that Caco-2 cells both attach to and proliferate on the hydrogels. Alamar Blue assay results of Caco-2 cells seeded on TCP at concentrations ranging from 5,000 cells/mL (1,200 cells/sample) to 285,000 cells/mL (68,400 cells/sample) yielded a calibration curve between fluorescence and cell count in cells per sample (Figure B.1.1). The Alamar Blue assay was performed on flat, crypt-like, and TCP samples, and the calibration curve equation was used to determine the cell count on the samples at Day 1. Using the raw data, cell counts on the TCP samples were calculated which suggested greater than 100% attachment to TCP. It was believed that a degree of cell death occurred between seeding Caco-2 cells on experimental samples and on the TCP calibration curve samples, resulting in Caco-2 cell concentrations being less than those specified in the calibration curve. In order to normalize the data to a maximum possible 100% cell attachment, the average fluorescence from TCP experimental samples was subtracted from calibration curve fluorescence values to produce a normalized standard curve equation (Figure B.1.2). Using this equation, the raw data was converted to a normalized Day 1 cell count that yielded a maximum percent attachment of 103.8 ± 9.9%. The normalized data suggest that Caco-2 cells demonstrate no more than 22.5% and 25.1% attachment.

<table>
<thead>
<tr>
<th>Seeding Density</th>
<th>Day 1 Cell Count (cells/sample)</th>
<th>Percent Attachment (%)</th>
<th>Normalized Day 1 Cell Count (cells/sample)</th>
<th>Normalized Percent Attachment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat Hydrogels</td>
<td>53,700</td>
<td>26,240 ± 14,670</td>
<td>48.9 ± 27.3</td>
<td>12,096 ± 14,670</td>
</tr>
<tr>
<td>Crypt-Like Hydrogels</td>
<td>96,135</td>
<td>38,240 ± 9,056</td>
<td>39.8 ± 9.4</td>
<td>24,096 ± 9,056</td>
</tr>
<tr>
<td>TCP</td>
<td>68,400</td>
<td>85,116 ± 6,772</td>
<td>124.4 ± 9.9</td>
<td>70,972 ± 6,772</td>
</tr>
</tbody>
</table>

Table 2: Calculated cell counts and percent attachment for Caco-2 cells seeded on flat hydrogels, crypt-like hydrogels, and TCP. Normalized data is based on a maximum possible percent attachment of 100%.
attachment to flat and crypt-like silk hydrogels, respectively (Table 2).

To further assess Caco-2 proliferation on the experimental samples, Alamar Blue assay was performed at time points over 21 days. At each time point, recorded fluorescence values on experimental samples are normalized to acellular controls, and the normalized value is divided by the Day 1 normalized fluorescence. With a baseline fluorescence compared to Day 1 of 1.0, values greater than 1.0 indicate proliferation, and values less than 1.0 indicate cell death.

The results of the Alamar Blue assay (Figure 14) indicate that Caco-2 cells proliferate on the surface of flat and crypt-like silk hydrogels and on TCP until reaching confluence. In the first three days, there is no significant increase in fluorescence, with crypt-like and TCP samples experiencing a decrease in fluorescence. By Day 5, there seems to be an upward trend in fluorescence, with crypt-like and TCP samples returning to approximately 1.0 and flat samples increasing to 1.6. At the Day 12 time point, all samples have a change in fluorescence greater than 1.0, indicating that the cells have proliferated. Also at the Day 12 time point, cells seem to have reached a plateau in proliferation. The change in fluorescence for flat, crypt-like, and TCP samples level out at approximately 1.7, 1.2, and 1.6, respectively. This asymptotic behavior suggests that cells have reached confluence between the Day 12 and Day 17 time point, as, once the cells have formed a confluent monolayer, contact inhibition prevents the cells from continuing to proliferate.

3.4. Cell Morphology on Hydrogels

Confocal microscopy with cell visualization by fluorescent staining allowed for the gross morphology of Caco-2 cells on flat and crypt-like hydrogels to be assessed. Hoechst visualization of the cell nuclei (blue) and Calcein AM visualization of the cell cytosol (green) on flat and crypt-like hydrogels 21 days after Caco-2 cell seeding confirm the previous Alamar Blue conclusion that
cells have reached confluence by this time point (Figure 15). Despite blue background fluorescence from Hoechst staining of the silk hydrogel, it is evident from the top view (top) that cells have formed a monolayer across the imaged region of the hydrogels. Furthermore, the cross-sectional side views (bottom) suggest cell orientation along the topography of the hydrogel. On the flat hydrogel (Figure 15A), the cross section indicates that cells have oriented along the flat surface of the gel, as expected. On the crypt-like hydrogel (Figure 15B), the cross section through the crypt shows that cells orient along the top surface of the hydrogel and along the surface within the crypt. Staining along the sides and towards the bottom of the crypt demonstrate that cells tend to migrate into the crypts and crevices of the hydrogel.

This finding shows that Caco-2 cells attach and can be grown on both flat and crypt-like hydrogels that have been 3D-printed, as well as that cells tend to align along the surface and in the crypts of the hydrogels. In order to model the in vivo intestinal tissue in a physiologically relevant manner, however, the in vitro cell layer must be confluent, align itself along the crypts, and function as a selectively permeable epithelial barrier. Future work is therefore needed to determine if confluent cell monolayers and mature, functional cell layers can be formed. In particular, the
quality of the cell layer must be further assessed with measurements of TEER, which will confirm the integrity and permeability of the monolayer and thus indicate whether the cell layer functions as an intestinal epithelial barrier. As a result, although these images suggest a baseline understanding of Caco-2 cell morphology on hydrogels, further understanding is needed before concluding whether the hydrogel model can more accurately model the in vivo large intestinal epithelium than traditional two-dimensional cell culture models.

3.5. Tight Junction Integrity on Hydrogels

To assess the integrity of tight junctions in the Caco-2 monolayer grown on flat and crypt-like hydrogels in the absence or presence of TNF-α, immunofluorescence staining of the tight junction protein, claudin-1, was performed. Caco-2 monolayers treated with complete media (Control) or complete media containing 10 ng/mL TNF-α for 48 hours were stained with anti-claudin-1 antibody and visualized with confocal microscopy (Figure 16). In the control condition, both flat and crypt-like gels display localization of claudin-1 protein to the cell membrane, between cells, although nonspecific intracellular staining is also visible (Figure 16A). Furthermore, there is continuous staining of claudin-1 protein around the cells, which indicates that tight junctions form a barrier between cells. When treated with TNF-α, however, both topographies of hydrogel show disruption of the tight junction barrier (Figure 16B). For the flat gel treated with TNF-α, the claudin-1 signal is noticeably diminished, with only single cells fluorescing in a single panel. In the cells that are visualized by the immunostaining (denoted by an arrow in Figure 16B), the staining of claudin-1 around the cell membrane is discontinuous. In the crypt-like gel treated with TNF-α, the claudin-1 signal is visible from the monolayer of cells surrounding the crypt, but it is weaker than that observed in the control sample. The claudin-1 staining also lacks the distinct localization to the cell membrane and orderly staining pattern that was present under normal conditions, as the claudin-1 visualization presents as a broad signal that appears to penetrate into the cell. The tight junction staining around the cell is also discontinuous, often manifesting as individual points rather than a continuous line.

Immunofluorescence imaging therefore suggests that Caco-2 cells grown on flat and crypt-like silk hydrogels display the tight junction integrity that is characteristic of an intestinal epithelial layer and that TNF-α treatment disrupts these tight junctions. These findings are consistent with literature, which suggests that TNF-α treatment decreases the tight junction integrity of the Caco-
2 cell layer to produce a phenotype that is characteristic of Crohn’s disease. However, in order to confirm that the Caco-2 cells grow into a functional monolayer with intact tight junctions, future work must include measurements of TEER across the monolayer. This method of analysis can also be used to quantitatively assess the effect of TNF-α on the tight junction barrier. If TNF-α does disrupt the tight junction barrier, as has been suggested by immunofluorescence staining, then the resistance across the Caco-2 monolayer would be less in the TNF-α-treated experimental group than the control group. This additional analysis would lend more validity to the conclusion.

Figure 16: Immunofluorescence staining of claudin-1 tight junction protein in Caco-2 cells on flat and crypt-like 4% (w/v) silk hydrogels. Cells were treated for 48 hours with (a) control media or (b) media with 10 ng/mL TNF-α. Imaged at 40X magnification with confocal microscopy.
that TNF-α disrupts tight junction integrity, especially due to the fact that differences in claudin-1 staining between normal and TNF-α conditions may be due to external factors, such as inconsistencies in the staining protocol or variations in the rate that cells metabolize the fluorescent stain. Thus, measuring TEER would be a reliable method to confirm whether TNF-α treatment successfully disrupts the tight junction barrier to produce a diseased phenotype.

3.6. Wound Healing on Hydrogels

Having developed a wound scratch assay protocol and demonstrated both quantitatively and qualitatively that Caco-2 cells attach to and proliferate on hydrogels to form a confluent monolayer, the next experimental aim, to assess the feasibility of using silk hydrogels as a model for intestinal epithelial cell wound healing, could be addressed. Epithelial restitution is a crucial process whereby the intestinal epithelium restores its barrier function after injury. To accurately model the process in vitro, a three-dimensional culture substrate that allows cells to form into the native crypt-like structure is necessary. It was therefore anticipated that the study of crypt-like hydrogels would facilitate wound healing that resembles the in vivo process.

Before conducting a wound healing study on flat and crypt-like hydrogels, the wound scratch assay procedure was repeated with Caco-2 cells on casted gels to test whether the protocol could be applied to wound healing on hydrogels. Imaging of the wound scratch on 2% (w/v) silk hydrogels casted at a 450-μm thickness suggests that the wound closure on the hydrogel resembles that observed on TCP (Figure 17). A wound scratch with defined edges is visible with sufficient contrast to analyze wound closure over the 4-hour period. The images also indicate that inducing the wound scratch in the cell layer with a pipette tip also scratched the gel surface, so it was noted

![Figure 17: Preliminary wound healing study with Caco-2 cells on casted flat 2% (w/v) silk hydrogels with a 450-μm thickness, imaged at 10X magnification.](image-url)
that care must be taken when scratching the hydrogels. With these findings, it was concluded that
the wound scratch procedure optimized for wound healing on TCP can also be used for wound
healing on hydrogels.

Caco-2 cells on flat and crypt-like gels cultured in the absence and presence of TNF-α were
scratched with a pipette tip, and the wound scratch was imaged at time points over 24.5 hours.
Representative samples imaged at $t = 0h$ and $t = 24.5h$ are depicted in Figure 18. Despite rotation
of the gel in the well over the 24.5-hour period, the same wound scratch area is present in the
image frame, allowing the degree of cell migration to be assessed (Figure A.2.1). For the flat and
crypt-like normal samples, minimal cell movement is noted over 24.5 hours. Cells are observed in
the lower left corner of the flat normal sample and to the right of the imaged crypt in the crypt-like
normal sample, in the location of the original wounds. For the TNF-α samples, there is no
observable cell movement.

These images indicate that there is a minor degree of two-dimensional cell movement, but the
most prominent observation from the images is that the wound scratch and cell movement are
difficult to visualize on the gel. One likely explanation for such difficulty in observing cell
movement is the method of imaging. By focusing on one plane of the hydrogel, it is not possible
to observe three-dimensional cell movement into or out of crevices, crypts, and other non-planar
regions of the gel. Cell movement to close the wound is not necessary two-dimensional, and since
even the flat surfaces of the gels are not perfectly flat, it is possible that cell movement towards

![Figure 18: Wound healing study with Caco-2 cells on 3D-printed flat and crypt-like 4% (w/v) silk hydrogels, cultured without (Normal) or with 10 ng/mL TNF-α (TNF-α). Representative samples depicted, imaged at 10X magnification.](image-url)
the wound scratch area is taking place in planes outside of the image focus. Another possible explanation is that cell movement may not be within the image frame. Since the wound scratch extends beyond the imaged region, it is possible that cells were moving to other non-confluent regions of the hydrogel.

Based on this wound healing study on hydrogel, it appears that both two-dimensional and three-dimensional cell movement may occur in response to a wound scratch. However, due to limitations in the imaging method that prevent cells on different planes of the gel from being in focus, limited wound closure is observed. In order to draw further conclusions about wound healing on flat and crypt-like hydrogels, confocal microscopy should be used to prepare a three-dimensional projection of the hydrogel using Z-stack images.
4. **Conclusions and Next Steps**

4.1. **Conclusions**

The findings of this study indicate that 3D-printed silk hydrogels are a suitable substrate for Caco-2 cell culture. A shape fidelity study first demonstrated that 3D-printed flat and crypt-like silk hydrogels retain their shape for 8 weeks in cell culture. Although statistically significant decreases in side length and hole diameter were observed, these changes were less indicative of a decrease in shape fidelity than the increased difficulty in handling noted with alginate hydrogels. Pending future work that will assess the reproducibility of the changes in side length and hole diameter, it can be concluded that silk hydrogels retain their shape and are thus suited for long-term cell culture. Flat and crypt-like silk hydrogels were also shown to support the attachment and proliferation of Caco-2 cells. Based on assays of metabolic activity and confocal microscopy of morphology and tight junctions, it also appears that Caco-2 cells proliferate into a confluent monolayer with a continuous tight junction barrier. Caco-2 cells were also shown to align along the crypts in crypt-like gels, such as is seen in the crypt-like epithelial layer. However, future work must be conducted to confirm that the Caco-2 cells grew into a confluent, functional monolayer.

Through this work, progress was also made towards developing an *in vitro* model for studying wound healing in the large intestine. The wound healing study on TCP confirmed that a 48-hour treatment with 10 ng/mL TNF-α is sufficient to simulate impaired wound closure that is characteristic of the Crohn’s disease state. Staining of the tight junction molecule claudin-1 also suggests that TNF-α alters tight junction integrity. However, future work that includes measuring TEER across Caco-2 cell layers grown in the absence and presence of TNF-α will be able to confirm whether TNF-α impairs wound healing by disrupting the tight junction barrier. When this optimized wound scratch assay protocol was applied to wound healing on flat and crypt-like hydrogels, it appeared that wound closure occurs both two-dimensionally and three-dimensionally. Due to limitations in the method used to image the wound scratch, however, it is not possible to draw conclusions as to the effect of topography or TNF-α on wound closure. As a result, the method of imaging wound healing on hydrogels must be further optimized to allow for visualization of both horizontal and vertical cell movement.

4.2. **Next Steps**
In order to further justify the use of 3D-printed silk based hydrogel as a culture substrate for Caco-2 cells, further shape fidelity studies must be performed. The acellular study performed must be repeated to investigate whether the statistically significant decrease in side length and hole diameter is reproducible. For a more in-depth assessment of shape fidelity, another shape fidelity study should be conducted with Caco-2 cells seeded onto the scaffold. Since enzymatic by-products produced by Caco-2 cell metabolism may increase the rate of silk hydrogel degradation, a cellular shape fidelity study would give a better indication of whether shape changes will occur over the course of a three-week cell culture period. Once the tendency for the hydrogel to undergo shape changes has been fully assessed, the hydrogel design can be scaled up during the 3D-printing process to account for these shape changes.

Furthermore, although it was shown in this work that Caco-2 cells attach and proliferate on 3D-printed flat and crypt-like silk hydrogels, the cell layer formed must be assessed for confluence, tight junction integrity, and epithelial barrier function. To do so, confocal microscopy with visualization of tight junctions can be used, in conjunction with measurements of TEER over the course of a 21-day cell culture period. A confluent monolayer of Caco-2 cells with intact tight junction molecules would demonstrate functionality by showing a plateau in TEER measurements as the cells reach confluence, as well as by recording values of transepithelial resistance consistent with literature values. This method of analysis can also be used to assess the decrease in tight junction integrity that occurs as a result of TNF-a treatment, as a Caco-2 cell monolayer with disrupted tight junctions would have lower TEER values than the control conditions.

As mentioned above, wound healing studies on these confluent Caco-2 monolayers should be repeated in healthy and diseased states with newly optimized methods of wound imaging. By using confocal microscopy and preparing Z-stack projections of the wound area, two- and three-dimensional cell movement to close the wound gap will be observed, rather than being restricted to observing cell movement within the plane of focus.

After optimizing the model of Caco-2 cells on silk hydrogels, the model be further developed into one that can recapitulate the complexity of the in vivo large intestinal epithelium. Although the topography of the crypt-like gel may facilitate the formation of a cell layer that resembles the in vivo crypts, the intestinal epithelium in the body consists of different cell types, including mucus-producing goblet cells, mucus, basal lamina, and more complexity than a single epithelial layer. As a result, in order to use this hydrogel as an in vitro model to study physiology and
pathophysiology of the large intestinal epithelium, further modifications must be made.

Due to the tunability of the silk hydrogel, it is believed that this model can serve as a baseline for a more holistic model of the intestinal epithelium. In particular, current work involves 3D-printing silk hydrogels with encapsulated fibroblasts. Interaction of the seeded Caco-2 cells with a second cell type would allow for wound healing and morphology to be more accurately represented, as the cell-cell signaling could affect cell motility. Furthermore, introduction of goblet cells into the cell layer would help support mucus production. The findings of this thesis project can therefore be built upon in the future to develop silk hydrogels into an in vitro model for understanding the physiology and pathophysiology of the large intestinal epithelium.
Acknowledgements

This Honors and University Scholar thesis project is sponsored by the Connecticut Regenerative Medicine Fund (15-RMA-UCONN-02) and the Office of Undergraduate Research Summer Undergraduate Research Fund Award. Thank you to Dr. Kelly Burke, Julia Tumbic, and Danielle Heichel, as well as faculty and fellow students in the Institute for Material Science for your mentorship, support, guidance, and camaraderie the past 2.5 years. Thank you Dr. Christopher O’Connell for your microscopy assistance and accommodation. Thank you to my family and friends for your support and encouragement throughout this entire research and thesis process.
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Appendix A – Annotated Images

A.1. Shape Fidelity Study

![Flat and Crypt-Like Silk Hydrogels](figure.png)

*Figure A.1.1:* Annotated image of shape fidelity study with 3D-printed flat (left) and crypt-like (right) silk hydrogels. Measurements for side length (SL) and hole diameter (HD) are depicted.

A.2. Wound Healing on 3D-Printed Hydrogels

![Wound Healing Study](figure2.png)

*Figure A.2.1:* Annotated image of wound healing study with Caco-2 cells on 3D-printed flat and crypt-like 4% (w/v) silk hydrogels, cultured without (Normal) or with 10 ng/mL TNF-α (TNF-α). Representative samples depicted, imaged at 10X magnification. Black boxes represent crypts. White lines represent edges of wound scratch. Black arrows depict direction of cell movement.
Appendix B – Supplemental Data

B.1. Cell Attachment and Proliferation

Figure B.1.1: Calibration curve relating fluorescence at 540 nm excitation and 590 nm emission to the number of cells per sample.

Figure B.1.2: Calibration curve relating fluorescence at 540 nm excitation and 590 nm emission to the number of cells per sample, normalized to a maximum possible attachment of 100%. 

Equation: $y = 0.0153x + 24.527$

$R^2 = 0.983$