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Cellular Attachment on Poly(Lactic Acid) for Use in Tissue Engineering

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CELLULAR ATTACHMENT ON POLY(LACTIC ACID) FOR USE IN TISSUE ENGINEERING

by

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Stephen R. Hunter, M.S.E.

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Tissue engineering is a promising new method for organ regeneration. This method can be used to repair damaged tissue, or possibly replace a fully functional organ. In summary, a biopsy is taken from a donor, isolated, then grown onto a framework resembling an organ. Once grown; it is then transplanted into the patient. Over time, the body will degrade the scaffold, leaving the organ or tissue in its place. As this scaffold is present in the body for an extended time; factors like biocompatibility, toxicity, immunogenicity, and structural stability must be researched.

Several polymers have already been researched for use in tissue engineering. However, one in particular, Poly(lactic acid) or PLA, has taken notice as this has been proven effective in current bone fixation procedures. Some medical devices like screws, pins, rods, meshes and plates already use this material and is approved by the FDA. The reason PLA is used is it degrades into lactic acid, an already present chemical byproduct found in the human body which will not stimulate a negative immune response. Current research shows several studies involving the use of osteoblasts but information on other cell types are extremely limited.

This thesis aims to investigate if cellular adherence is possible on this hydrophobic polymer using several different methods and three-dimensional printing orientations. H4iie cells were cultured on six acid-catalyzed PLA scaffolds for four days. Each scaffold was either uncoated or coated with extracellular matrices Matrigel or Collagen IV. Cellular attachment was then quantified and compared to the control group.

Results show cellular attachment was possible on an untreated surface. However, due to the hydrophobicity of the polymer, a protein substrate with a vertically printed surface enhanced cellular attachment. As this material is currently being used in medical procedures, PLA may be a suitable, nontoxic material that has great potential for further research in tissue engineering.

ACKNOWLEDGMENTS

I would like to begin by acknowledging the influence of my graduate advisor, Dr. James Springstead. Without your guidance in both undergrad and graduate classes, I would not be where I am today. Your enthusiasm and support of academic work inspired me to push myself and pursue this thesis.

Secondly, I would like to thank my peers that assisted me in the preliminary research, Piao Jian Tan, and the mechanical engineering department for providing me with the Poly(lactic acid) scaffolds. Thirdly, I want to thank all members of my thesis committee, Dr. James Springstead, Dr. Andrew Kline, and Dr. Qiang Yang for taking the time to review my work.

Lastly, I would like to thank my wife, Chelsea, for having the patience to stand by my side to see this through. I especially want to thank my godmother, Sherry, without whom I would not be where I am today. Your support and guidance have helped more than you can imagine.

Throughout this process it has been an excellent experience even with the serious outbreak of Covid-19. This work was slightly impacted due to the closure or Western Michigan University laboratories. One set of experiments were completed but as labs were shut down for nearly a year, no repeat replicates were performed. This would provide a statistical significance to these results.

Stephen R. Hunter

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LIST OF ABBREVATIONS

3D	Three-Dimensional
ECM	Extracellular Matrix
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal Bovine Serum
FDA	Federal Drug Administration
H&E	Hematoxylin & Eosin
HEK	Human Embryonic Kidney
MEM	Minimum Essential Medium
PBS	Phosphate Buffered Saline
PCL	Poly(caprolactone)
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
UV	Ultraviolet

INTRODUCTION

Overview

Tissue engineering is a fairly new advancement in biomedical science. Typical procedures focus on extracting a biopsy from either a compatible donor or an individual's own cells. These are then grown and cultured in a monolayer using a tissue culture flask or petri dish. Cells are then expanded using several growth factors to enhance cellular proliferation. This is because primary cells are terminally differentiated. Grown cells are then transferred and cultured onto a three-dimensional scaffold that is made out of a biocompatible polymer that acts as a framework for cells to adhere to and proliferate around. Once a fully functional organ is created, it is then transferred into the patient's body. Refer to Figure 1 for a simplified diagram explaining this procedure.

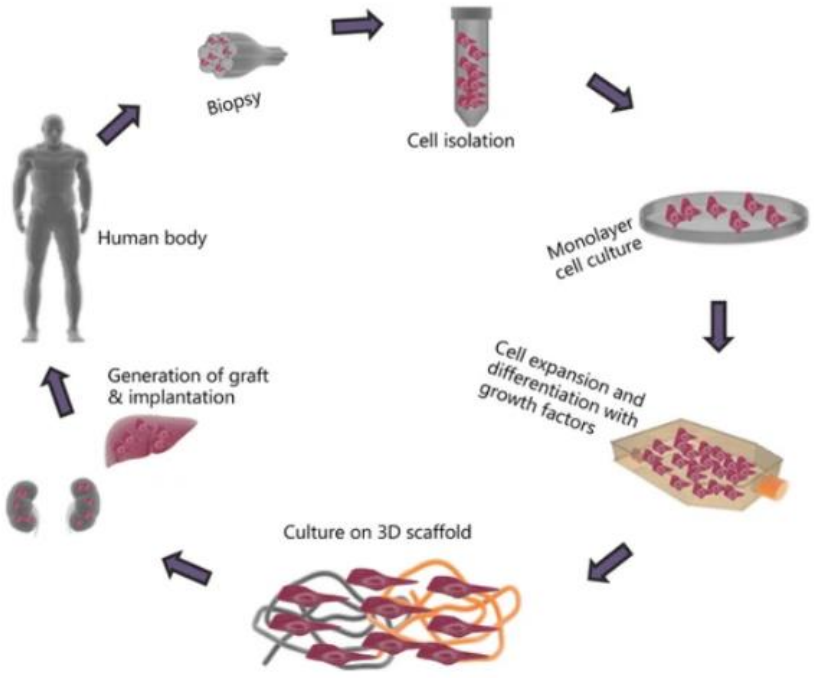


Figure 1: Tissue Culture Engineering Diagram (Ude, 2018)

Several factors must be considered and researched before developing a scaffold in tissue engineering. First and foremost, the scaffolding must be made of a biocompatible and nontoxic material that can sustain this for long term exposures. Cells must be able to adhere and function normally on the polymer surface and throughout the entirety of the scaffold. Cells must also be able to function normally, grow, proliferate and repair themselves before and after implantation without eliciting a negative immune system response. These scaffolds must also be degradable and its products must not elicit a negative immune response. Scaffolds should also have a high porosity and an interconnected pore structure that ensures cells are adequately supplied with nutrients (O'Brien, 2011).

Several different materials and polymers have already been investigated in the field of tissue engineering. Some polymers that have already been approved by the FDA are Poly(lactic acid), poly(glycolic acid), and poly(caprolactone). Poly(caprolactone) or PCL is a rubbery type polymer that loses its integrity after several years. This is especially not suitable for tissue regeneration as this can be harmful for long term exposures. Poly(glycolic acid) is another polymer used in various clinical applications. However, the lifespan of this material in the human body is very short lived. PGA is typically broken down in as little as one month. PLA is an ideal polymer as this can be broken down in as few as a couple months to a year, refer to Figure 2. This degradation may be due to its hydrophobicity. Poly(lactic acid), or PLA, is a "biocompatible, biodegradable, and immunologically inert synthetic polymer" (Tagle, 2010). It is an extremely hydrophobic material compared to that of PGA which makes this polymer more suitable for tissue engineering.

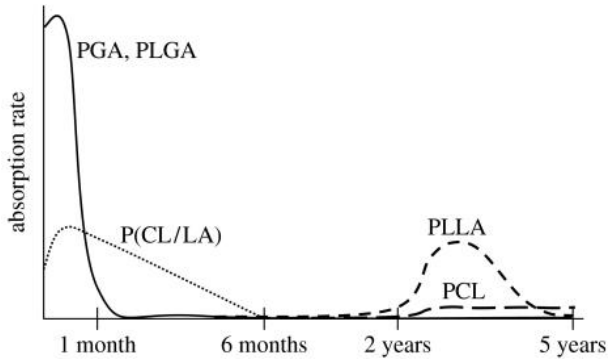


Figure 2: Adsorption Comparison (Ikada, 2006)

Poly(lactic acid) has already been approved by the Federal Drug Administration (FDA) for use in various wound management and bone fixation applications, which makes this ideal as this has been deemed healthy from the FDA and the legwork in terms of testing and procedures has already been completed. Current medical procedures and devices that currently use PLA are sutures, stents, screws, pins, and grafts. As this polymer breaks down, it degrades into lactic acid, an already present chemical by product found in red blood and muscular cells in the human body. Due to this, this material is a great option since it will not stimulate a negative immune response.

Hydrophobicity

PLA is a hydrophobic polymer that can affect cellular adherence. As there is a carbonyl group and methyl group located on the chain, refer to Figure 3 for a molecular diagram of PLA. There is a lack of reactive side-chain groups the make this polymer inert. This inertness prevents the polymer from reacting with water (Baran, 2019). Because of this hydrophobicity, several cell lines may have difficulty in adhering to the surface of a scaffold.

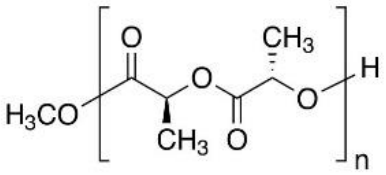


Figure 3: Poly(Lactic Acid) Molecule, (polysciences.com)

Degradation

One of the major contributors to this degradation reaction is hydrolysis. In the presence of water, the diffusion of water into the polymer hydrolytically cleaves the ester groups located on the main chain, refer to Figure 4 for a simplified degradation reaction of PLA. This then forms water soluble compounds that make it easier for metabolic clearance.

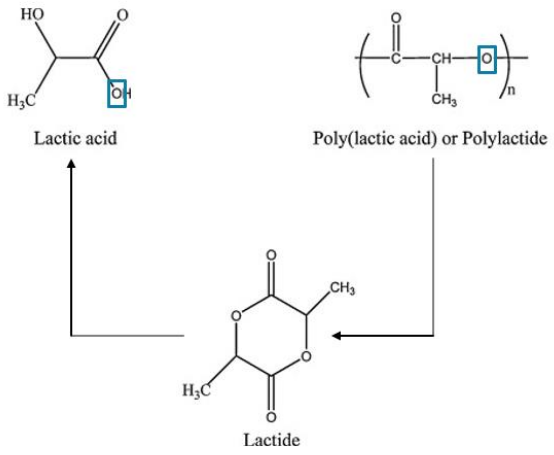


Figure 4: Degradation Reaction

Temperature and the pH of the hydrolytic degradation can also significantly increase or decrease the clearance time at which the body degrades the polymer. Temperature can have a significant effect on the hydrolytic degradation of PLA, especially at higher temperatures. However, at body temperature the clearance constant can be estimated close to one hundred thousand times slower than that of 220 °C which is very slow, refer to Figure 5. To determine this constant, Gorrasi used a modified derivation of the Arrhenius equation with an Activation Energy of 10000 K. As this is an extremely high activation energy, this is the cause of the kinetic constant being so low as shown in Figure 5.

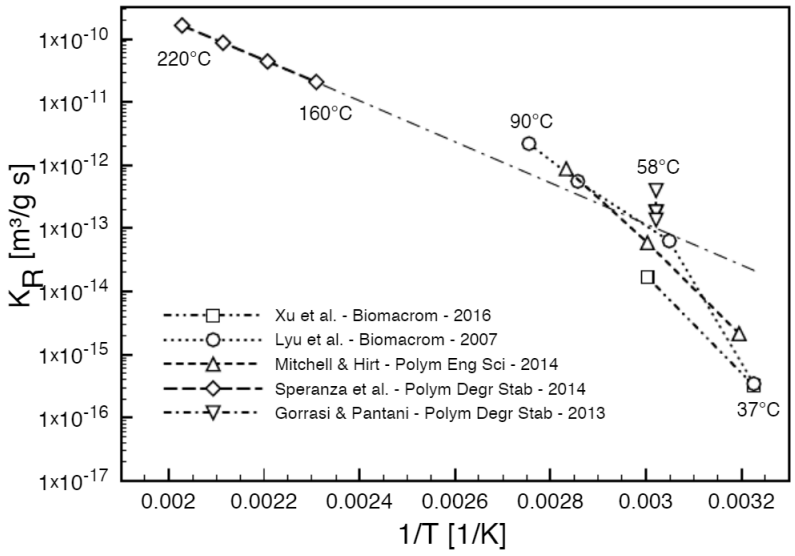


Figure 5: Temperature Degradation (Gorrasi, 2013)

Another factor that should be considered is the pH of the area the transplant will take place. Depending on the area, pH can vary significantly, refer to Figure 6. For reference, stomach acid is highly acidic with a pH of around 1 to 2 and the pH of blood is somewhere in the middle of basic and acidic at around 7.4-7.6 (Gorrasi, 2013). Refer to Figure 6 to see the normalized kinetic constant calculated by Gorrasi. In essence the kinetic constant is highly

dependent on the pH of the system. This data can assist in determining the degradation of PLA in certain areas of the body. Transplanting a newly functional organ into areas of the body that is very acidic, or in areas with a pH greater than 5 is optimal.

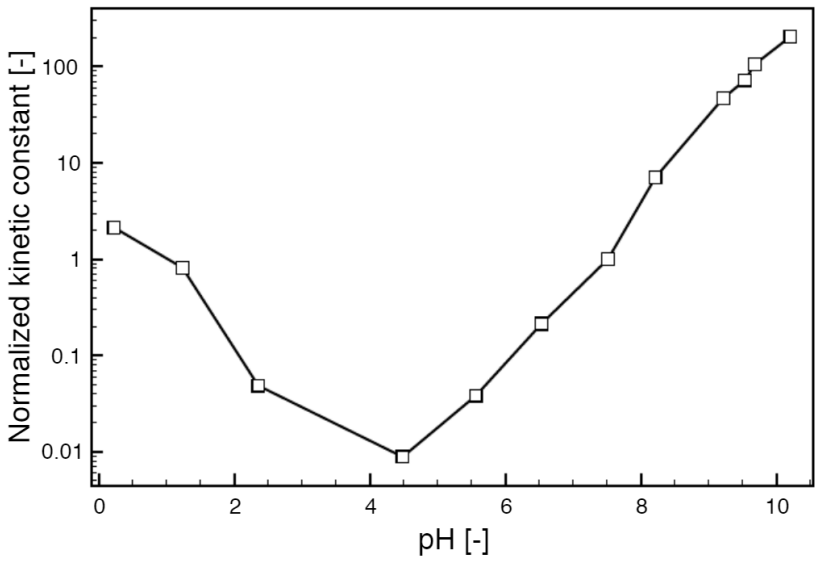


Figure 6: pH Degradation (Gorrasi, 2013)

Protein Substrates

To enhance cellular attachment, protein substrates are typically used in cell culture practices as an attachment factor. "Attachment factors are structural proteins or protein-like substances that have adherent capabilities and increase cell-substrate interactions in a culture dependent attachment milieu. A number of glycoproteins have been identified that promote and/or influence in-vitro cell attachment to the surface or substratum of the culture vessel. Normal attachment, growth and development of many cell types are dependent on attachment factors and extracellular matrix (ECM) components. While some cells are able to synthesize these components, others require an exogenous source" (Biological Industries).

Collagen I, Rat Tail is a fibrous protein abundant in connective tissues such as the tendon, ligament, dermis, and blood vessels. It is the major component and the primary determinant of tensile strength of the extracellular matrix (ScienCell). Another protein substrate that is commonly used in cellular attachment is matrigel, or commonly known as basement membrane extracellular matrix. Matrigel is a combination of extracellular proteins and growth factors like collagen, laminin, and gelatin (Corning). This proprietary concoction of extracellular matrix proteins makes it a suitable candidate for cellular attachment as this will provided the most diverse conditions for cellular attachment.

Porosity

Porosity of a 3-dimensionally printed culture vessel can also have a major impact on cellular attachment. The porosity or void fraction of the scaffold must be small enough to facilitate mechanical interlocking between the cellular tissue and the three dimensional scaffold. Porosity must be able to provide a structurally stable support for the weight of the tissue as well as be large enough to effectively transport cellular waste as well as biofactors like proteins, genes, and nutrients for cell growth. Pores should also provide enough room for vascularization or blood flow to develop. The difficulty in developing a scaffold is to develop a balance between the mechanical and mass transport function of the scaffolding system (Loh, 2013).

Three-Dimensional Printing

Three-dimensional printing orientation can create variable pores and channels. For the sake of this thesis, square three-dimensional scaffolds were printed with the following dimensions (35.4 mm x 35.4 mm x 2 mm). Refer to Figure 7 for an image displaying one printed

layer out of several hundred layers. Scaffolds printed in a horizontal orientation are printed from the outer most edges first then closer towards the center is a very porous and lattice structure. If looking at the scaffold from above and into one pore, the ability to see through is not possible. This is because each layer is not consistent with the layer below, leaving variable pore and channels. Another way to think about it is that the outer edges are very smooth and solid, while the center of the scaffold consists of a lattice pattern leaving larger pores. Scaffolds printed in a vertical orientation are printed in the same fashion except these are taller and printed vertically rather than horizontally. Outer most edges are printed first, and these are not porous and very smooth, but the center area consists of a lattice pattern leaving very small pores in the center. With the vertically printed scaffolds, cells will have a difficult time entering the scaffold as the outer most surfaces provide little to no porosity.

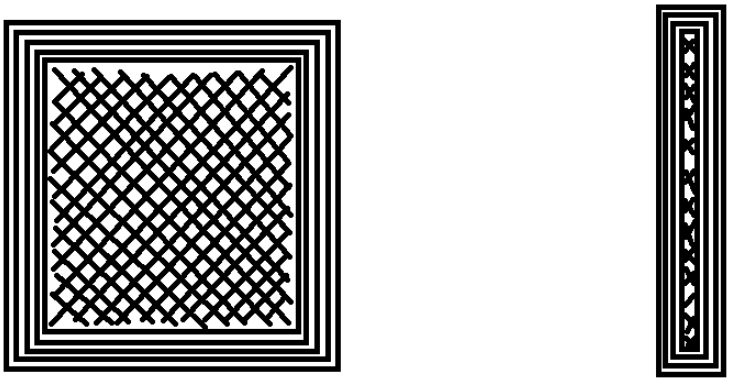


Figure 7: 3D Printing Protocol

Chemical Engineering Concepts

Current biological research has only recently started to explore using PLA for tissue engineering. As this is still in its infancy, research has been tailored to known practices like

bone tissue replacement procedures. Using chemical engineering principles, developing a cheap polymer like PLA for use in tissue engineering used the knowledge of mass transport, chemical kinetics of nutrients and polymers found in Organic Chemistry.

Gaps in Research

Poly(lactic acid) is easily decomposed in the body, leaving a void in its place. Current procedures use this polymer for bone screws, pins and devices that can easily decompose as the bone heals itself. However, there is a huge lack of available research using PLA with other cell types.

Experimental Objectives

1) Determine if a mammalian cell line can adhere on an untreated acid-catalyzed Poly(lactic acid) scaffold.
2) Determine if the same cell line can adhere on a protein substrate coated Poly(lactic acid) scaffold.
3) Determine if the printing orientation of the scaffold affects cellular adherence.

PRELIMINARY EXPERIMENTS AND RESULTS

Preliminary research was performed by a fellow graduate student, Piao Jian Tan. In his procedure, Human Embryonic Kidney, HEK-293, cells were cultured in 100 mm petri dishes and maintained in a 37 °C, 5% CO_2, humidified incubator with complete growth medium consisting of 90% Eagle's Minimum Essential Medium, 10% Fetal Bovine Serum and a 1%, 100x stock of Penicillin/streptomycin. Fresh medium was replaced every two to three days and cells were subcultured as needed in sterile petri dishes. Cells were then seeded onto square, acid-catalyzed Poly(lactic acid) scaffolds (35.4 mm x 35.4 mm x 4 mm) into glass 60 mm petri dishes. Post seeding, fresh medium was replaced every day as well as imaging, refer to Figure 8. Due to the thickness and opaqueness of the scaffold, visualization of cells was extremely difficult. After seven days in culture, a Hematoxylin and Eosin, H&E, stain was used to stain the cells for better visualization on the topmost portion of the scaffold.

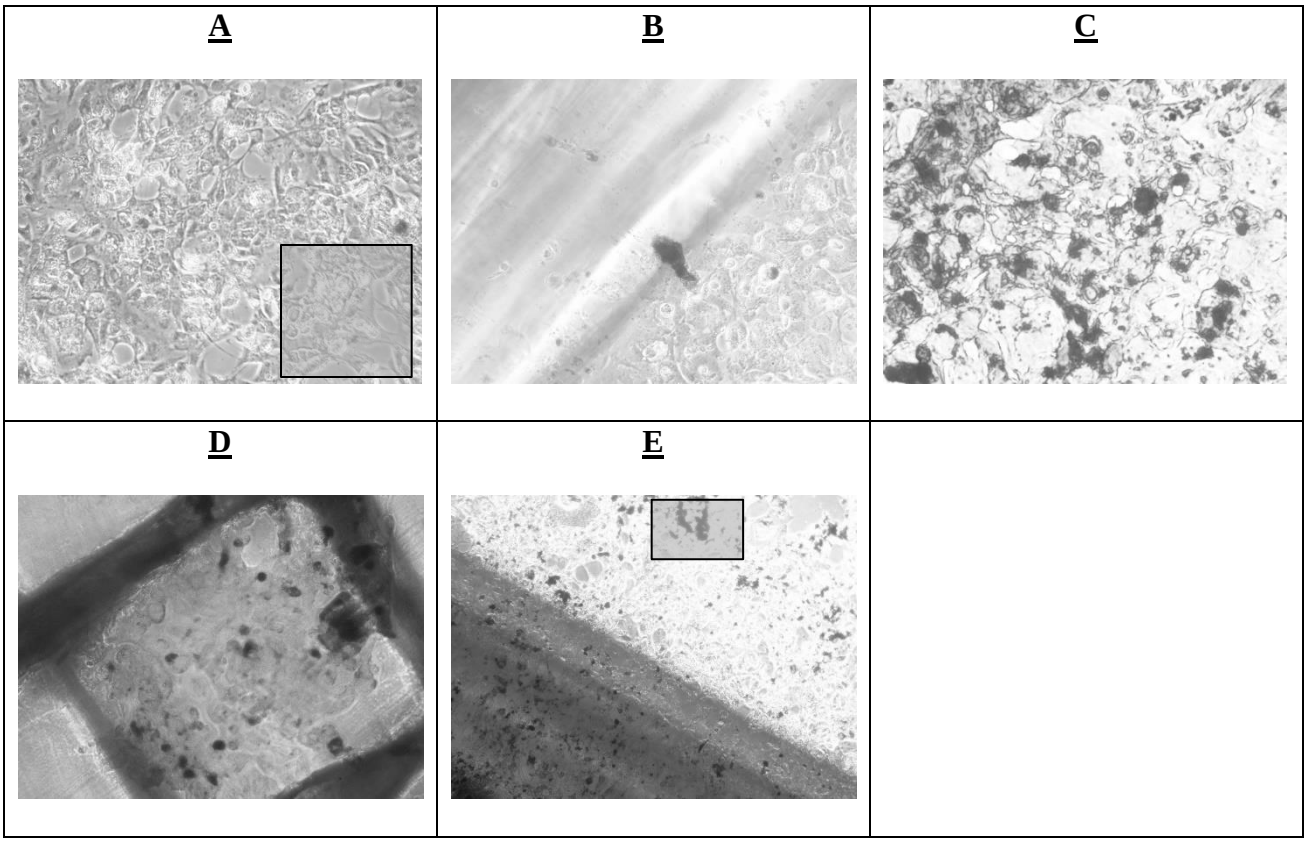


Figure 8: Preliminary Images, HEK-293 Cells (Tan, 2019)

Image A shows HEK-293 cells growing under normal conditions in a glass petri dish. Image B displays the topmost corner of the horizontal scaffold with HEK-293 cells attached to the petri dish. Image C is a higher resolution image of Image B. Image D displays one pore on the topmost section of the horizontally printed scaffold after the H&E stain. Dark spots indicate the cell nucleus. Image E shows the topmost corner of the horizontally printed scaffold after the H&E stain. Dark spots indicate cell nuclei. It was indicated from Piao Jian Tan, that cells were indeed attached to the topmost surface.

Preliminary images display that cellular attachment using this protocol is indeed possible. However, there was very limited cellular attachment across the entirety of the topmost surface.

Cells that did adhere were very sparse, separated and a higher concentration of cells tended to migrate, adhere and grow along the corners and edges of the scaffold.

As HEK-293 cells can be cultured in both monolayer and suspension conditions, this cell line is loosely adherent and should be changed. Upon taking leadership of this project another cell line was researched to determine if another cell line would enhance cellular attachment. It was determined to use H4iie cells. H4iie cells are a cheap, robust, and strongly adherent epithelial cell line derived from Rat liver, *Rattus Norvegicus.* This particular cell line is traditionally used in several in vitro cytotoxicity models to closely mimic human liver toxicity. They are an extremely robust cell line that has strong resistances and a doubling time of approximately "22-24 hours" (Yang, 2011) while HEK-293 cells can be grown in suspension or by attachment with a doubling time of "34 hours" (Cervera, 2011). Another minor consideration is that HEK-293 cells are derived from Human Embryonic Kidney's. As these are an embryonic cell line, this can be viewed as controversial.

H4iie cells were cultured and maintained in 150 cm^2 tissue culture flasks, (CytoOne, Cat# CC7682-4815) using a complete growth medium consisting of 90% Minimum Essential Medium (Gibco, Cat#: 11095-080, Lot: 2152915) and 10% Fetal Bovine Serum (Gibco, Cat#: 26140079, Lot: 1645615) in a 37 °C, 5% CO_2 humidified incubator. Prior to seeding, Poly(lactic acid) scaffolds were soaked in 70% ethanol for thirty minutes then sterilized under ultraviolet light for another 30 minutes. Then, 100 mm petri dishes were coated with a basement layer of 1% Agar, Cells were then seeded at a density of five million cells onto vertically printed square Poly(lactic acid) scaffolds in 100 mm petri dishes. Media was gently changed every day for four days. Pictures were taken daily using a 4X inverted microscope, refer to Figure 9.

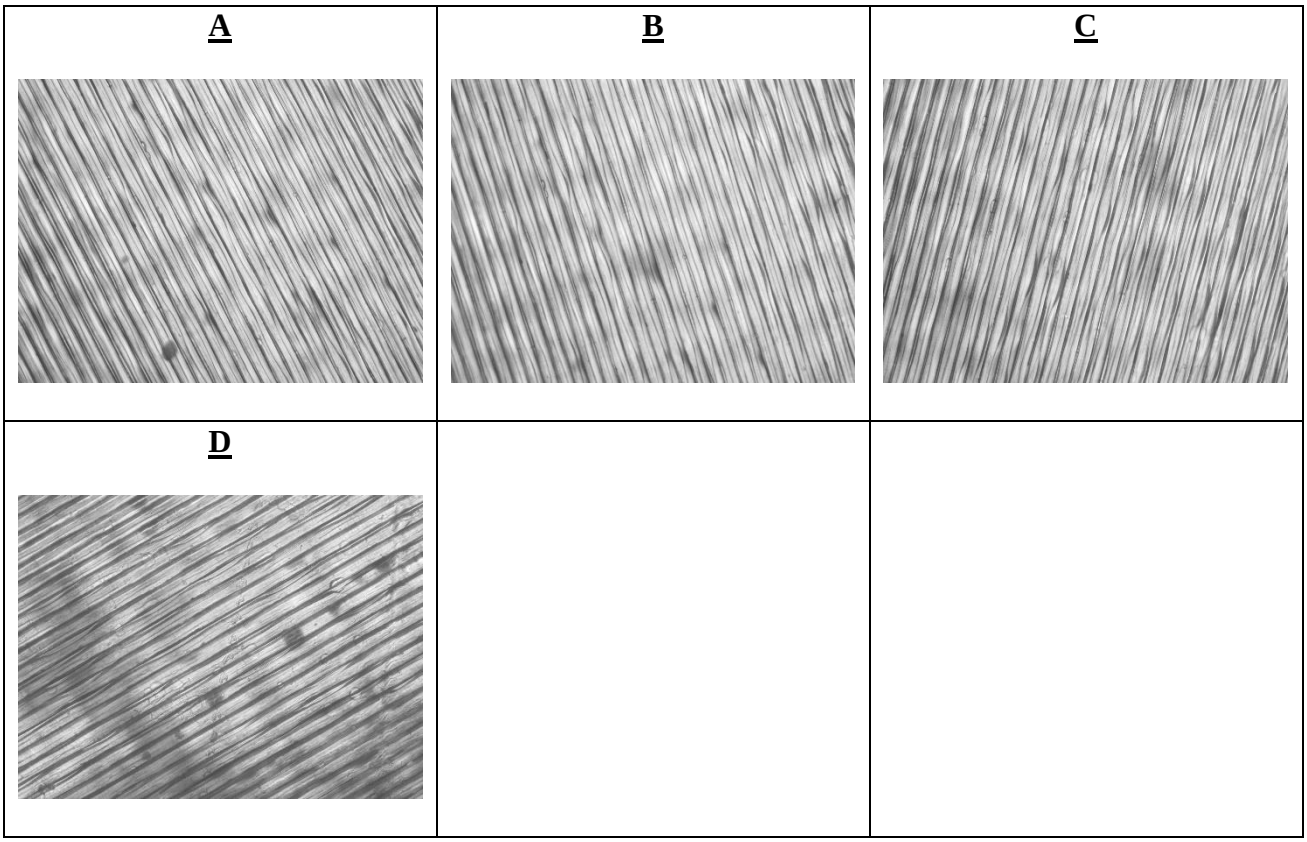


Figure 9: Preliminary Images, H4iiE Cells

Image A is a picture taken of the uncoated vertically printed scaffold post seeding. Image B is a picture taken of an uncoated, vertically printed scaffold one day after seeding. Image C is a picture taken of the uncoated, vertically printed scaffold three days after seeding. Image C is a picture taken of the uncoated, vertically printed scaffold four days after seeding. No distinguishable cell bodies were displayed in Images A, B or C. However, Image D displays small cell colonies of H4iie cells attached to the uncoated, vertically printed PLA scaffold. After accounting for the doubling time, these are not the ideal conditions for cellular adherence. However, these results show that cellular adherence is indeed possible; but this may be enhanced by using a protein substrate. Another variable that may enhance cellular attachment is the printing orientation of the scaffold.

METHODS

Three-Dimensional Scaffold Printing Procedure

Scaffolds were printed with the following dimensions, 35.4 mm x 35.4 mm x 2 mm. Scaffolds were printed at 220 °C, a 60 °C plate temperature and a 100 % infill. Once the scaffold was printed, the surfaces were polished with grinding papers of grade 600, 1200-fine then with a 1-micron grade containing alumina. After polishing, the scaffolds were washed with deionized water then 70% ethanol and air dried for 5 hours. Scaffolds were then sterilized for 10 minutes under an ultraviolet light (UV) before being soaked in deionized water. Then sterilization continued with being heated in a 130 °C oven for 30 minutes. To treat the surface, scaffolds were treated using an acid-catalyzed reaction by soaking in a 1 M sodium hydroxide and ethanol solution for 6 hours. After soaking, scaffolds were washed with deionized water thoroughly and vacuumed for 6 hours.

Cell Culture and Initial Procedure

H4iie cells (ATCC, Cat#: CRL-1548, Passage 30) were cultured and maintained in 150 cm^2 tissue culture flasks, (CytoOne, Cat# CC7682-4815) using a complete growth medium consisting of a 90% Minimum Essential Medium (Gibco, Cat#: 11095-080, Lot: 2152915) and 10% Fetal Bovine Serum (Gibco, Cat#: 26140079, Lot: 1645615) in a 37 °C, 5% CO_2 humidified incubator. Prior to seeding, Poly(lactic acid) scaffolds were soaked in 70% ethanol for thirty minutes then sterilized under ultraviolet light for another 30 minutes. Scaffolds were placed into a petri dish coated in a basolateral layer of 1% wt/vol Agar using the following experimental conditions, refer to Table 1. Only one replicate was performed for each experimental condition.

Table 1: Experimental Conditions

	No substrate	Matrigel (0.25 mg/mL)	Collagen I (50 µg/mL)
Vertical	1 scaffold	1 scaffold	1 scaffold
Horizontal	1 scaffold	1 scaffold	1 scaffold

Collagen I, Rat Tail Coating Procedure

Collagen I, Rat Tail was purchased from Gibco (Cat#: A10483-01, Lot#: 2103638, Exp: 2020-07-03, Storage: 4 °C, Concentration: 3 mg/mL). It was recommended from the supplier that a desired concentration of 50 µg/mL be used to coat the surface. To ensure enough substrate was used to entirely submerge the scaffold, 5 mL of substrate was made for each scaffold.

$$C_{Stock}V_{Initial} = C_{Final}V_{Final}$$

$$V_{Final,Collagen} = \frac{C_{Stock}V_{Initial}}{C_{Final}} = \frac{50\,\frac{\mu g}{mL} * 5\,\frac{mL}{scaffold} * 2\ scaffold}{3\,\frac{mg}{mL} * 1000\,\frac{\mu g}{mg}} = 0.1667\ mL$$

$$V_{20\ mM\ Acetic\ Acid\ in\ PBS} = \left(5\,\frac{mL}{scaffold} * 2\ scaffold\right) - 0.1667\ mL = 9.833\ mL$$

Equation 1: Collagen Formulation Calculations

The collagen solution was prepared on ice by transferring 166.7 µL of Collagen IV to a cold 15 mL conical containing 9.833 mL of ice cold, 20 mM Acetic Acid, refer to Equation 1 for calculations. Each scaffold was submerged into 5 mL of prepared substrate and incubated at room temperature for 1 hour. The solution was then removed using an aspirator connected to a vacuum and the scaffolds were allowed to air dry before use.

Matrigel Coating Procedure

Matrigel, or basement membrane ECM was purchased from Corning (Cat# 354277, Lot#: 354277, Storage: -80 °C, Concentration: 10 mg/mL). From previous experiences, typical concentrations of matrigel for primary hepatocytes was 0.25 mg/mL. To ensure enough substrate was used to entirely submerge the scaffold 5 mL of substrate in complete growth media was made for each scaffold.

$$C_{Stock}V_{Initial} = C_{Final}V_{Final}$$

$$V_{Final,Matrigel} = \frac{C_{Stock}V_{Initial}}{C_{Final}} = \frac{0.25\,\frac{mg}{mL} * 5\,\frac{mL}{scaffold} * 2\ scaffold}{10\,\frac{mg}{mL}} = 0.25\ mL$$

$$V_{Media} = \left(5\,\frac{mL}{scaffold} * 2\ scaffold\right) - 0.25\ mL = 9.75\ mL$$

Equation 2: Matrigel Formulation Calculations

The matrigel solution was thawed overnight at 4 °C in an ice bath. As this a thermo-sensitive gel, once this is exposed to heat the proteins will polymerize and solidify. Once thawed, 250 µL of matrigel was transferred to an ice cold 50 mL conical containing 9.75 mL of ice-cold media using ice-cold pipet tips, refer to Equation 2 for calculations. Each scaffold was submerged into 5 mL of prepared substrate then immediately placed in a 37°C, humidified incubator overnight.

Agar Gelling Procedure

As agar is commonly used in microbiological culture and its ability to solidify and form as a hydrogel, this was used to hold the scaffold in place. To do this, agar powder was purchased on Amazon and manufactured by Now Real Food. A 1% wt./vol agar solution was prepared by

adding 5 grams of agar powder to 500 mL of deionized water in a glass autoclavable jar. Solution was whisked and mixed thoroughly before being heat treated and sterilized in a 121 °C autoclave for 25 minutes. Once the container was touchable with gloved hands, 20 mL of warmed agar was transferred to each 100 mm petri dish in a sterile Biosafety Cabinet. Once the agar solution began solidifying, each scaffold was immediately pressed into the hydrogel, exposing the topmost surface. Scaffolds and agar were allowed to rest at room temperature for 30 minutes in a biosafety cabinet until the solution solidified.

Cell Seeding Procedure

Media was removed from the tissue culture flask using an aspirator connected to a vacuum. H4iie cells were then washed with 15 mL of Phosphate Buffered Saline (PBS). PBS was removed using an aspirator and 4 mL of TrypLE (Gibco, Cat#: 1253011) was transferred to the flask using a serological pipette. The cell culture flask was incubated at 37°C for approximately 5 minutes until cells detached from the surface. 5 mL of warmed complete growth medium was added to the flask to neutralize the enzymatic digestion. The entire volume of cell suspension was transferred to a 15 mL centrifuge tube and centrifuged at 200xG for 8 minutes. Supernatant was removed by aspiration and the cell pellet was resuspended in 10 mL of fresh complete growth medium. 25 µL of cell suspension was immediately transferred to a 1.5 mL Eppendorf containing 25 µL of Trypan Blue (Hyclone, Cat#: SV30084.01). 10 µL of trypan blue cell suspension was transferred to each compartment on an automated cell counter slide. Cells were then counted using a Countess II automated cell counter, refer to Table 2 for direct output from the cell counter. In the left column, this is this direct output calculated using the automated cell counter. However, the cell counter does not consider the total volume of the

cell suspension. Since the total volume of the cell suspension was 10 mL, the left column was multiplied by 10 mL to obtain the Total Number of cells.

Table 2: Initial Cell Counts

	Cells/mL	Total Cells
Count 1	4.16 (10^6)	4.16 (10^7)
Count 2	3.85 (10^6)	3.85 (10^7)
Average	4.01 (10^6)	4.01 (10^7)

Cells were then seeded onto each scaffold at a seeding density of five million cells per scaffold in 5 mL of complete growth medium.

$$Volume\ of\ Cell\ Suspension = \frac{5000000\,\frac{cells}{scaffold} * 6\ scaffolds}{40050000\ cells} * 10\ mL = 7.5\ mL$$

$$Media\ Needed = \left(5\,\frac{mL}{scaffold} * 6\ scaffold\right) - 7.5\ mL = 22.5\ mL$$

Equation 3: Cell Seeding Calculations

7.5 mL of cell suspension was transferred to a sterile 50 mL centrifuge tube containing 22.5 mL of fresh complete growth medium. This was inverted several times then 5 mL of this cell suspension was transferred to each petri dish containing a scaffold. Each scaffold was incubated in a 37 ’C incubator for one hour to allow cells to settle and attach before an additional 15 mL of fresh medium was added to each dish to completely cover the surrounding surface.

Maintenance Procedure

Each scaffold was cultured in a 37 °C, 5% CO_2 humidified incubator. To maintain healthy cells nutrients 20 mL of fresh media replacement every day. This not only adds nutrients

to keep cells healthy, but this also removes any residual waste byproducts released from the cells.

Take Down Procedure

On Day 4, some scaffolds began lifting out of the agar gelatin. So, initiation of the take down procedure was started. Scaffolds were removed from their culture dish and transferred to a new petri dish. 3 mL of warmed TrypLE was added to each scaffold and these were allowed to incubate in a 37 °C, 5% CO_2 incubator for 5 minutes. Using a 1 mL pipette, the TrypLE was gently agitated on the scaffold to ensure adequate cellular detachment. Then, 5 mL of warmed complete growth medium was added to each petri dish to neutralize the enzymatic digestion. Each scaffold was then agitated using a serological pipette 3 times. The entire contents of each dish were then transferred to a separate 15 mL centrifuge tube and centrifuged at 200xG for 8 minutes. Supernatant was then removed using an aspirator connected to a vacuum, and the cell pellets were resuspended 5 mL of fresh complete growth medium. 25 µL of cell suspension was immediately transferred to a 1.5 mL Eppendorf containing 25 µL of Trypan Blue (Hyclone, Cat#: SV30084.01). 10 µL of the trypan blue cell suspension was transferred to each compartment on an automated cell counter slide. Cells were then counted using a Countess II automated cell counter and compared to their respective control group.

RESULTS

In this section, images were taken daily of each scaffold under a 4x inverted microscope. Several images were taken, and the best quality images were chosen to represent each experimental condition.

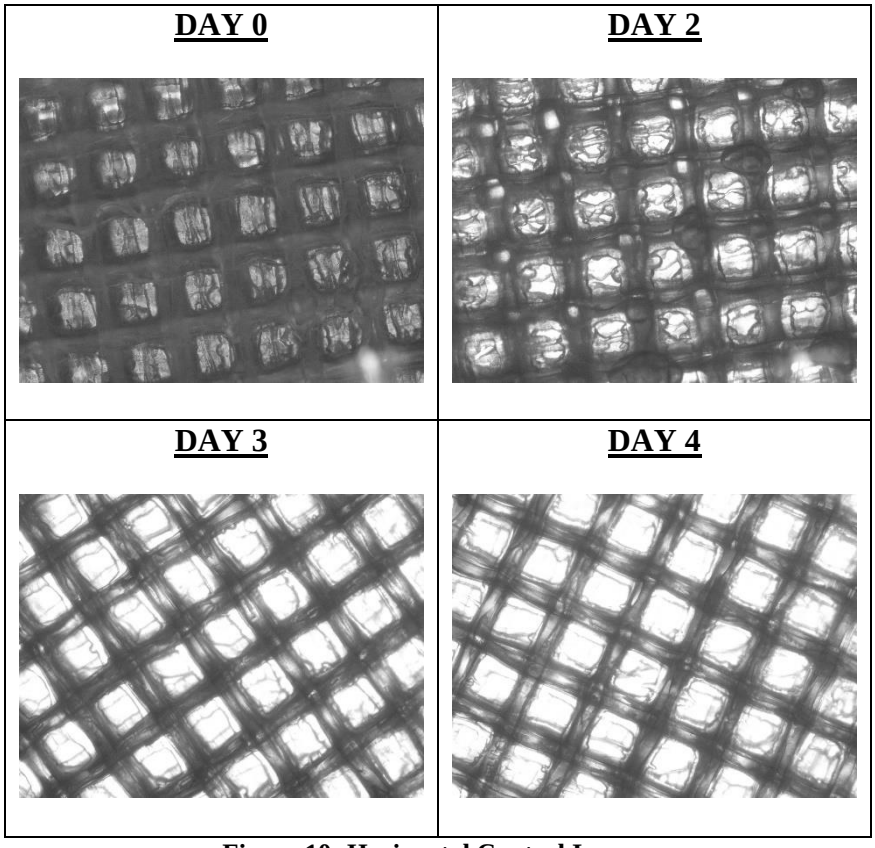


Figure 10: Horizontal Control Images

Refer to Figure 10 for images related to the uncoated horizontally printed scaffold. There are no distinguishable cell bodies visible in these photos. From these photos it is not clear what resides in each pore. However, it may be residual Agar being pushed into the scaffold during the setting process.

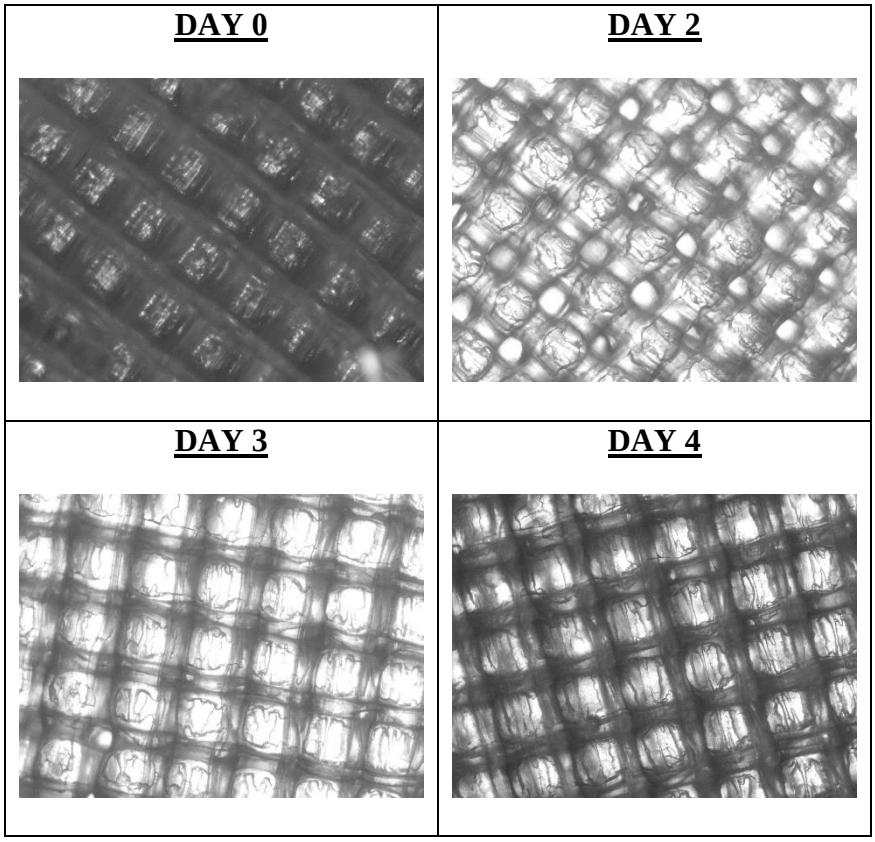


Figure 11: Horizontal Collagen Images

Refer to Figure 11 for images related to the Collagen coated horizontally printed scaffold. There are no distinguishable cell bodies visible in these photos. From these photos it is not clear what resides in each pore. However, it may be residual Agar being pushed into the scaffold during the setting process.

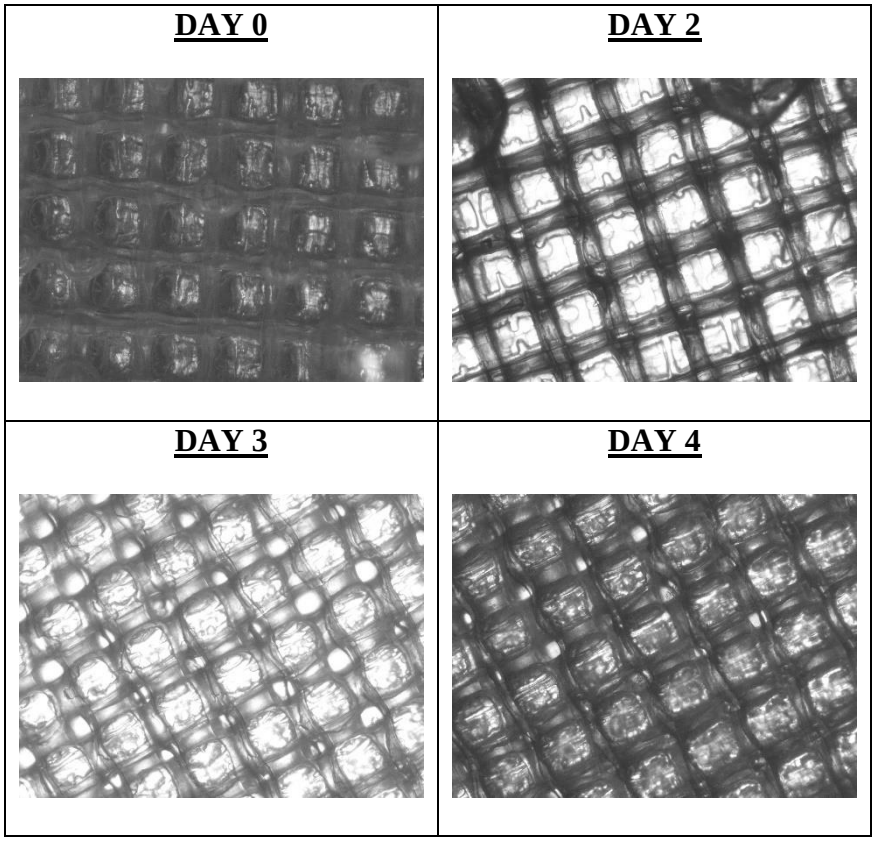


Figure 12: Horizontal Matrigel Images

Refer to Figure 12 for images related to the Matrigel coated horizontally printed scaffold. There are no distinguishable cell bodies visible in these photos. From these photos it is not clear what resides in each pore. However, it may be residual Agar being pushed into the scaffold during the setting process.

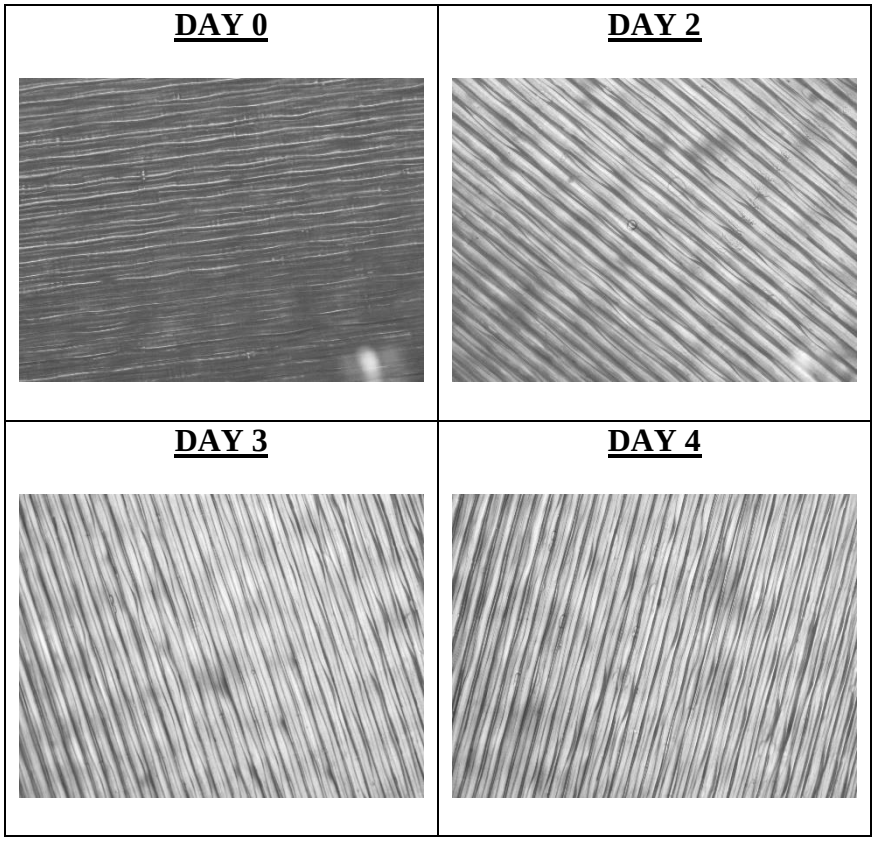


Figure 13: Vertical Control Images

Refer to Figure 13 for images related to the uncoated, vertically printed scaffold. There are no distinguishable cell bodies visible in these photos.

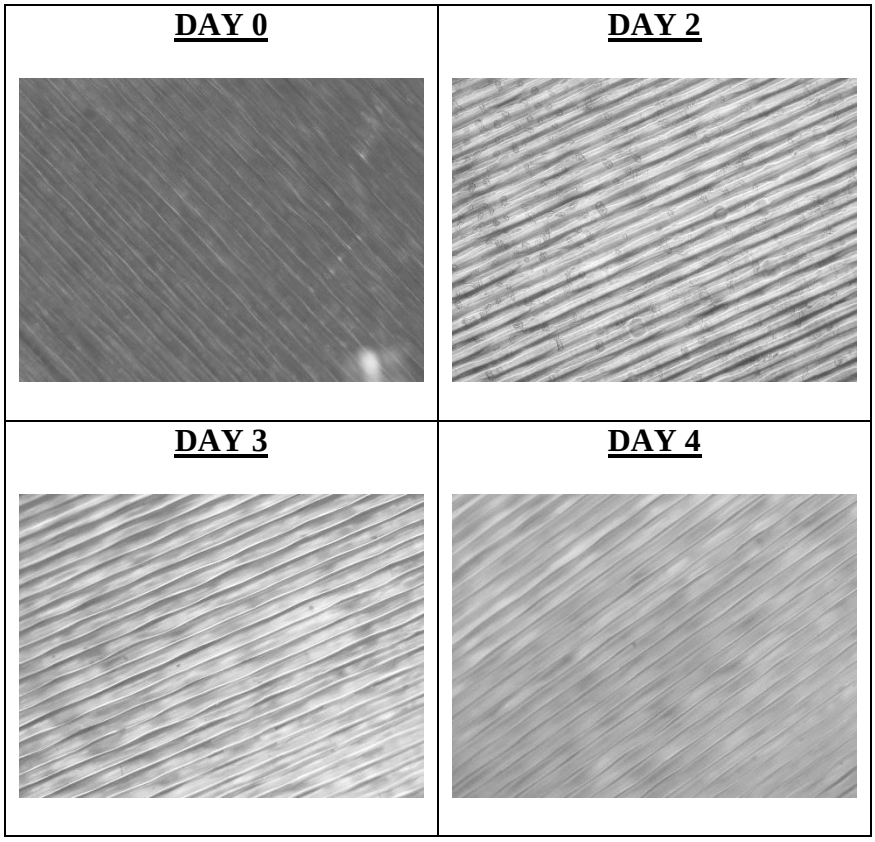


Figure 14: Vertical Collagen Images

Refer to Figure 14 for images related to the Collagen coated, vertically printed scaffold. Day 2 displays significant cell colonies and single cell structures attached to the surface of the collagen coated, vertical scaffold. On day 3, the shadows are cell colonies attached on the inner portion of the scaffold. On day 4, the image was extremely hazy as the scaffold lifted from the agar. Thus, the poor visual quality.

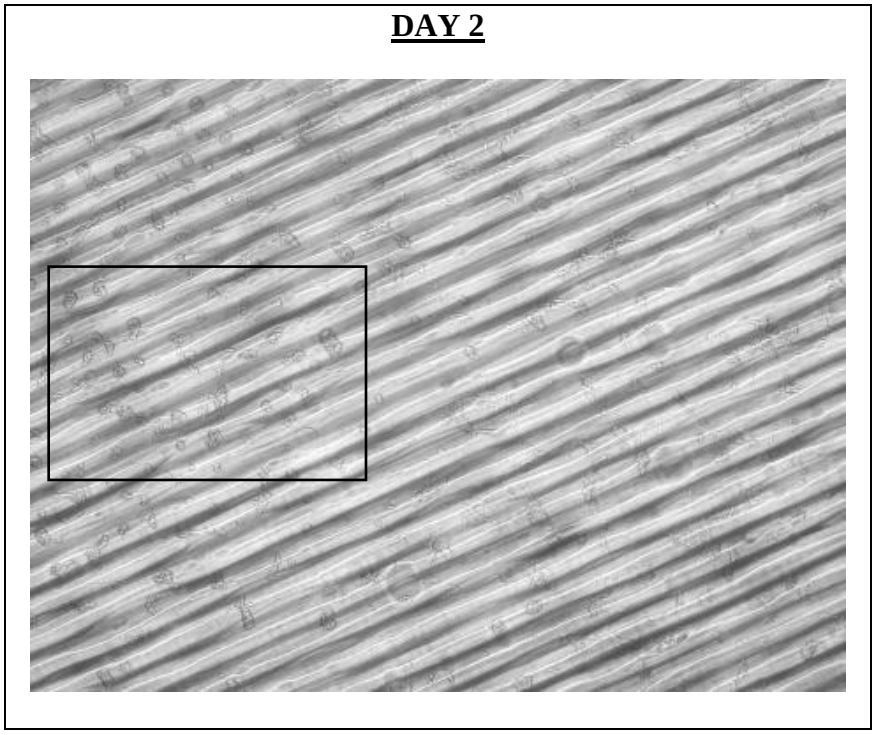


Figure 15: Vertical Collagen Image (Day 2)

Refer to Figure 15 for a larger image related to Day 2 of the Collagen coated, vertically printed scaffold. In this image, several cell colonies and single cell structure outlines are visibly attached to the scaffold surface. Cells tended to be very sparse through the entire top portion of the surface and there is limited cell networking occurring.

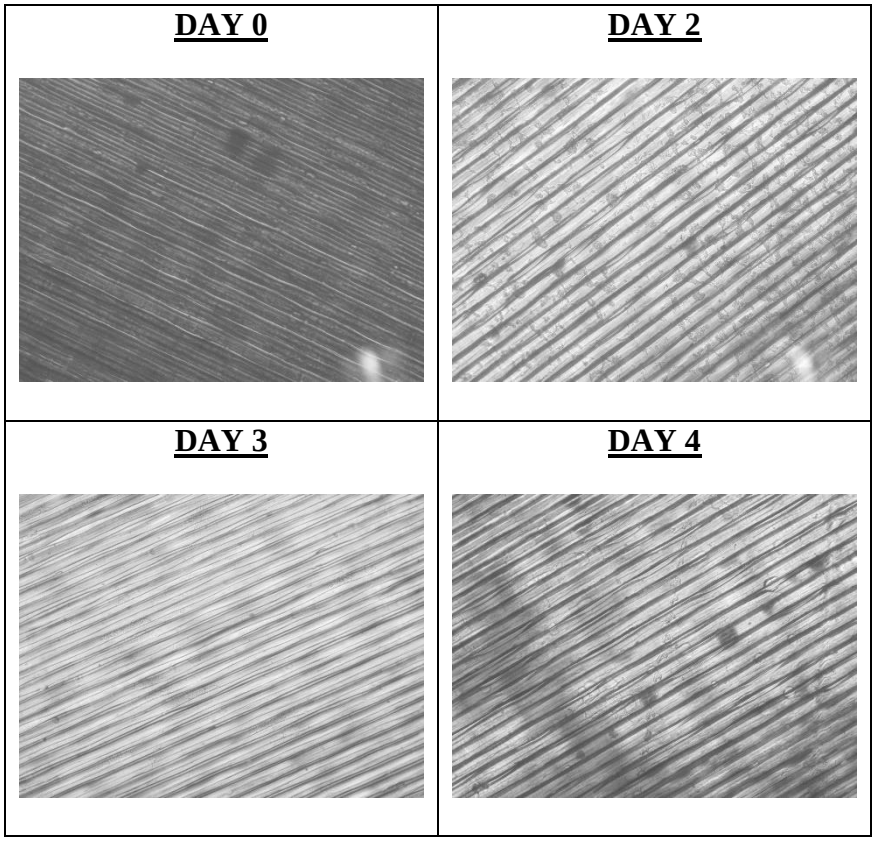
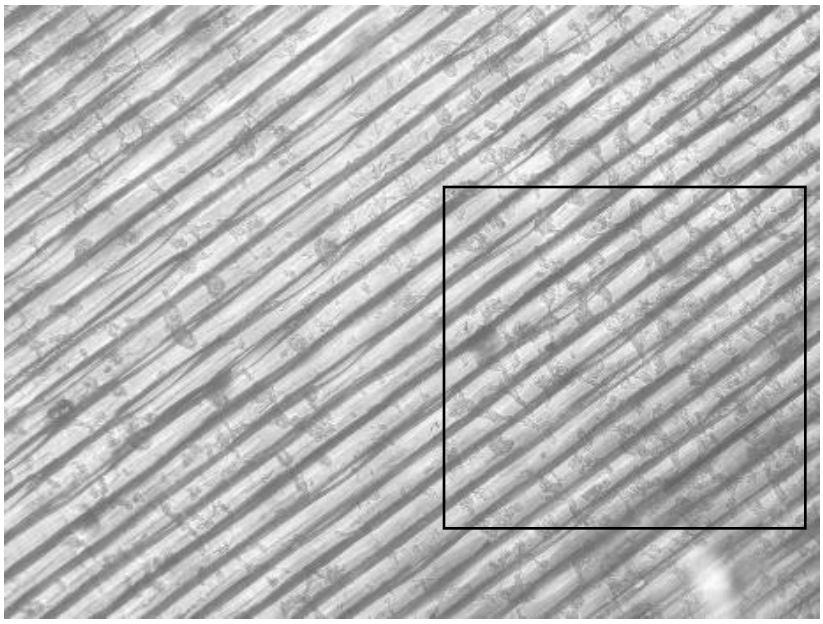


Figure 16: Vertical Matrigel Images

Refer to Figure 16 for images related to the Matrigel coated, vertically printed scaffold. Days 2, 3 and 4 display significant cell colonies and single cell structures attached to the surface of the matrigel coated, vertical scaffold.

DAY 2



DAY 3

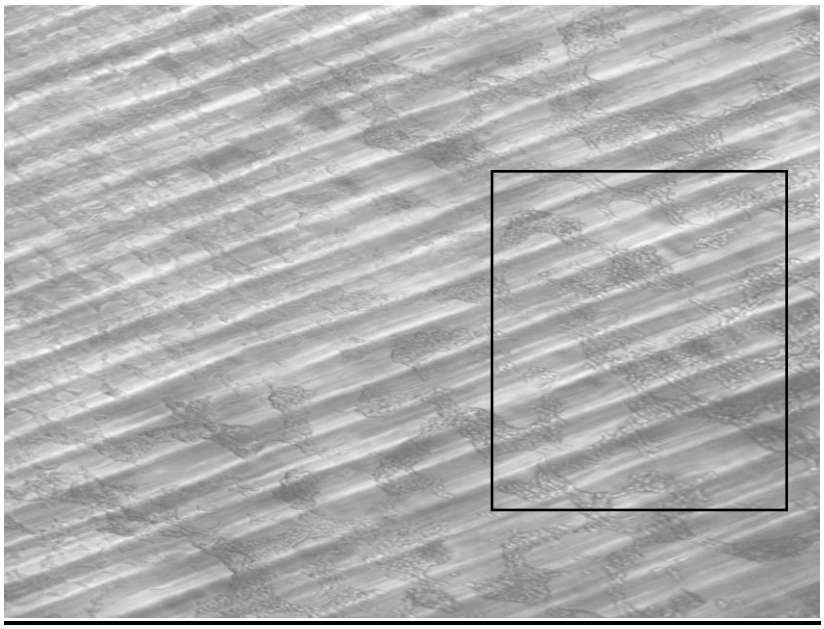


Figure 17: Vertical Collagen Image (Day 2)

Refer to Figure 17 for a larger image related to Day 2 and 3 of the Matrigel coated, vertically printed scaffold. In this image, several cell colonies and cellular networks are attached to the scaffold surface.

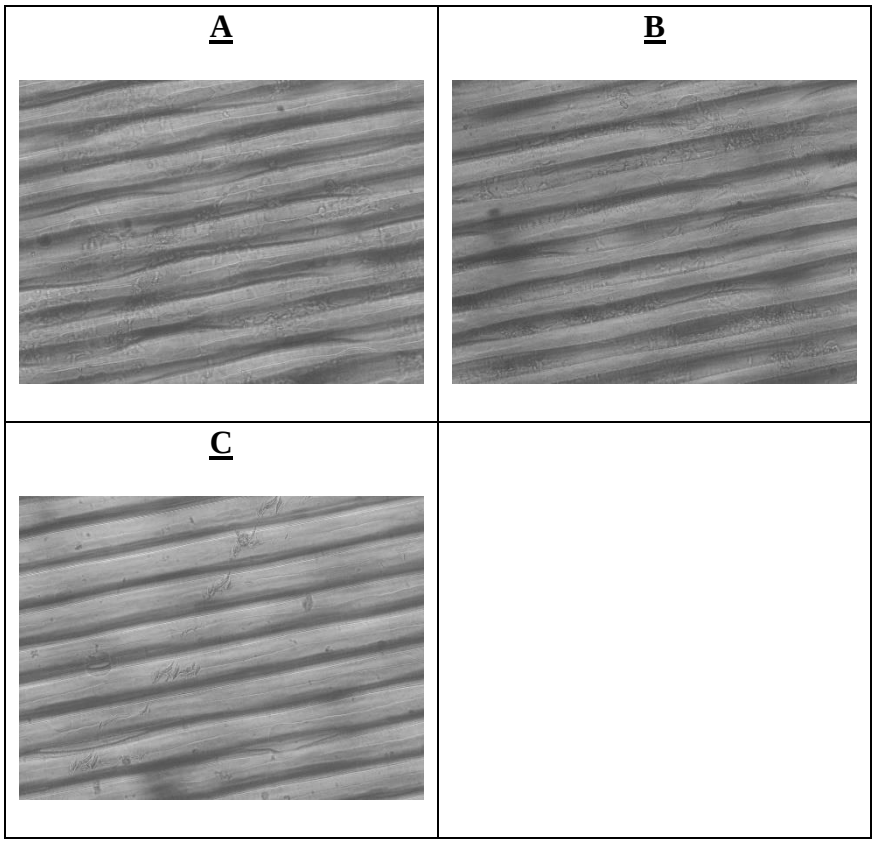


Figure 18: Vertical Matrigel Additional Images (Day 4)

Refer to Figure 18 for additional day 4 photos that were taken of the Matrigel coated, vertical scaffold. Using a 10X, inverted microscope, Image A displays evenly spread, cell colonies that have begun interconnecting and quite possibly forming an ECM. Image B is another image of evenly spread cell colonies. Image C displays a corner most section of the scaffold that does not display any cellular attachment.

Table 3: Final Cell Count Results

Horizontal (Control)		
	Cells/mL	**Total Cells**
Count 1	0	0
Count 2	35200	176000
Std Dev	-	88000
Average	17600	88000

Vertical (Control)		
	Cells/mL	**Total Cells**
Count 1	5860	29300
Count 2	3520	176000
Std Dev	-	73350
Average	4690	102650

Horizontal (Collagen I)		
	Cells/mL	**Total Cells**
Count 1	334000	1670000
Count 2	346000	1730000
Std Dev	-	30000
Average	340000	1700000

Vertical (Collagen I)		
	Cells/mL	**Total Cells**
Count 1	346000	1730000
Count 2	411000	2055000
Std Dev	-	162500
Average	378500	1892500

Horizontal (Matrigel)		
	Cells/mL	**Total Cells**
Count 1	5860	29300
Count 2	5860	29300
Std Dev	-	0
Average	5860	29300

Vertical (Matrigel)		
	Cells/mL	**Total Cells**
Count 1	469000	2345000
Count 2	575000	2875000
Std Dev	-	265000
Average	522000	2610000

Cells were counted using a Countess II automated cell counter, refer to Table 3 for direct output from the cell counter. In the left column, this is this direct output calculated using the automated cell counter. However, the cell counter does not consider the total volume of the cell suspension. Since the total volume of the cell suspension was 5 mL, the left column was multiplied by 5 mL to obtain the Total Number of cells.

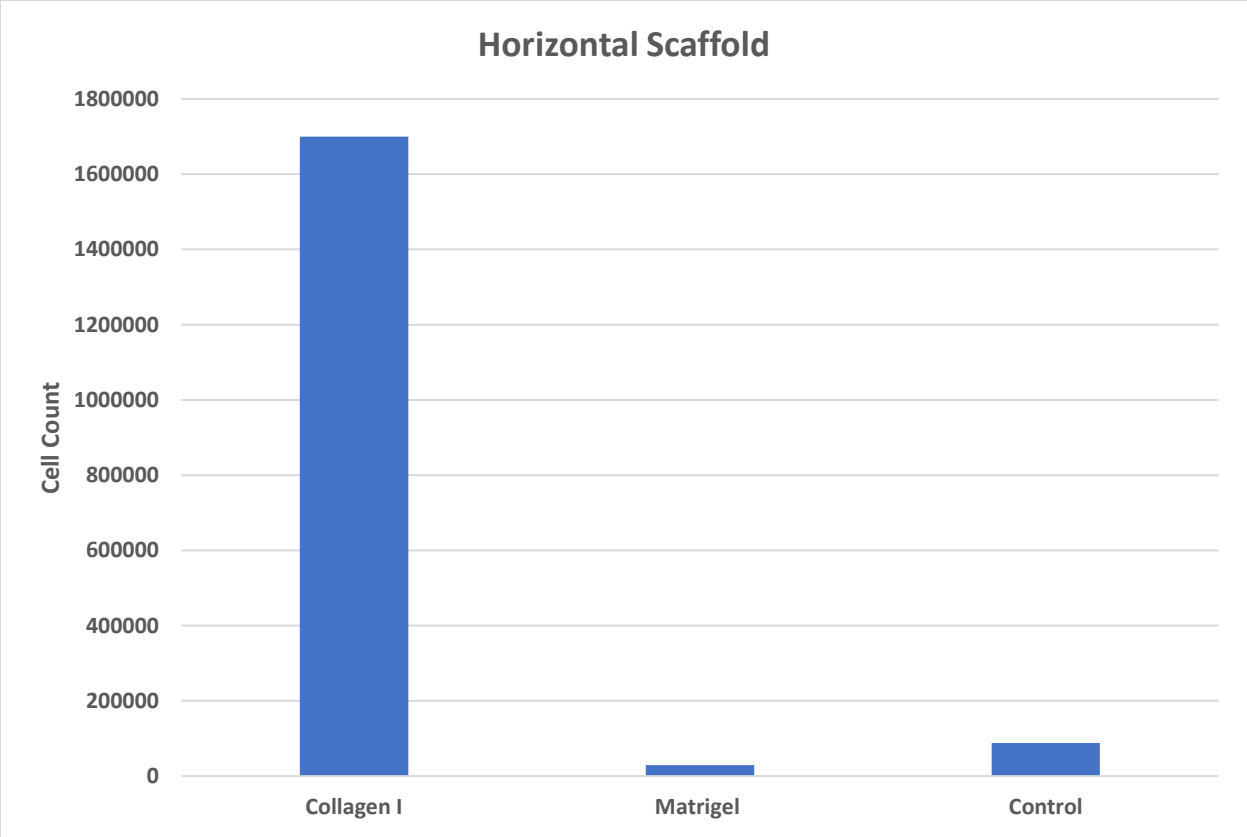


Figure 19: Horizontally Printed Scaffold Results

Total cells calculated from Table 3 were graphed using a bar chart for each horizontally printed experimental condition, refer to Figure 19. For the horizontally printed scaffolds, Collagen I provided extremely higher cell counts compared to the matrigel and control groups. Error bars are not provided as only one replicate was performed.

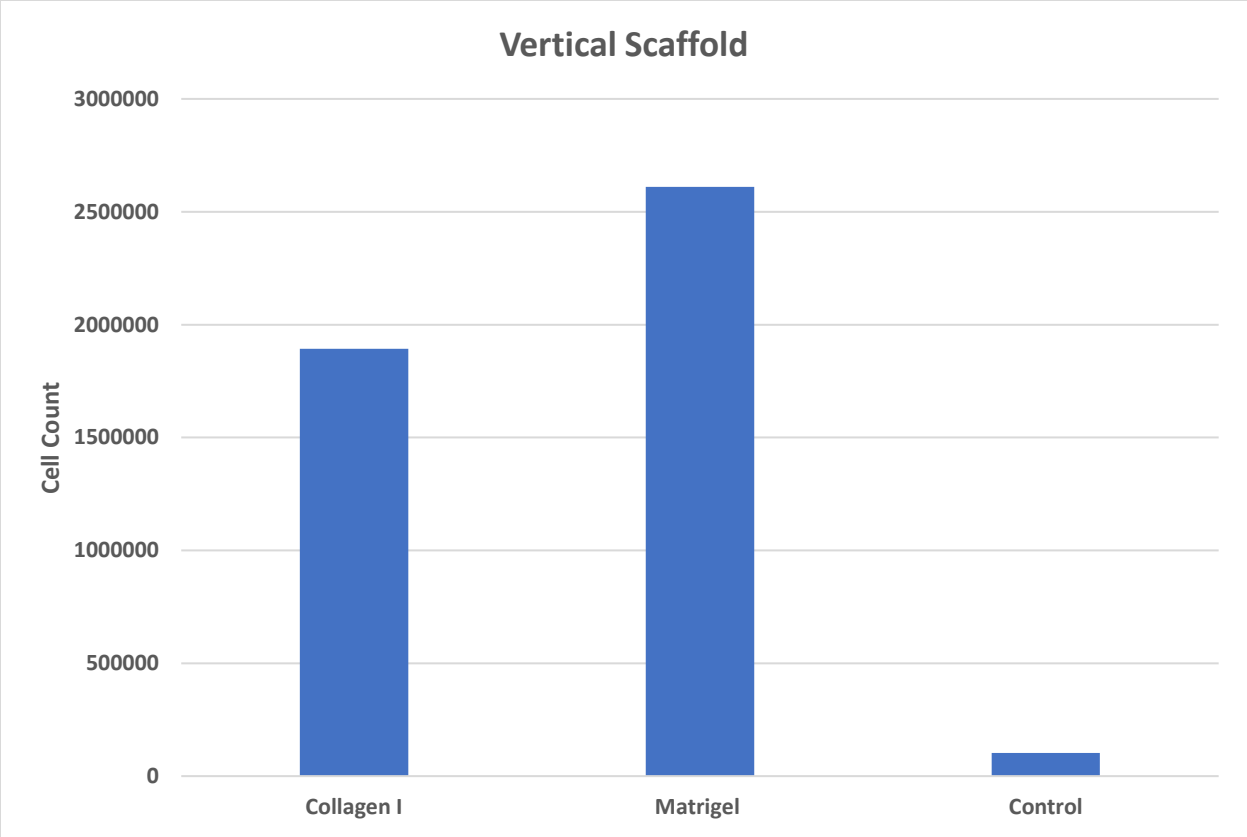


Figure 20: Vertically Printed Scaffold Results

Total cells calculated from Table 3 were graphed using a bar chart for each vertically printed experimental condition, refer to Figure 20. For the vertically printed scaffolds, Matrigel provided the highest cell counts compared to the Collagen and control groups. However, Collagen I provided similar results to the Matrigel coated scaffolds. Error bars are not provided as only one replicate was performed.

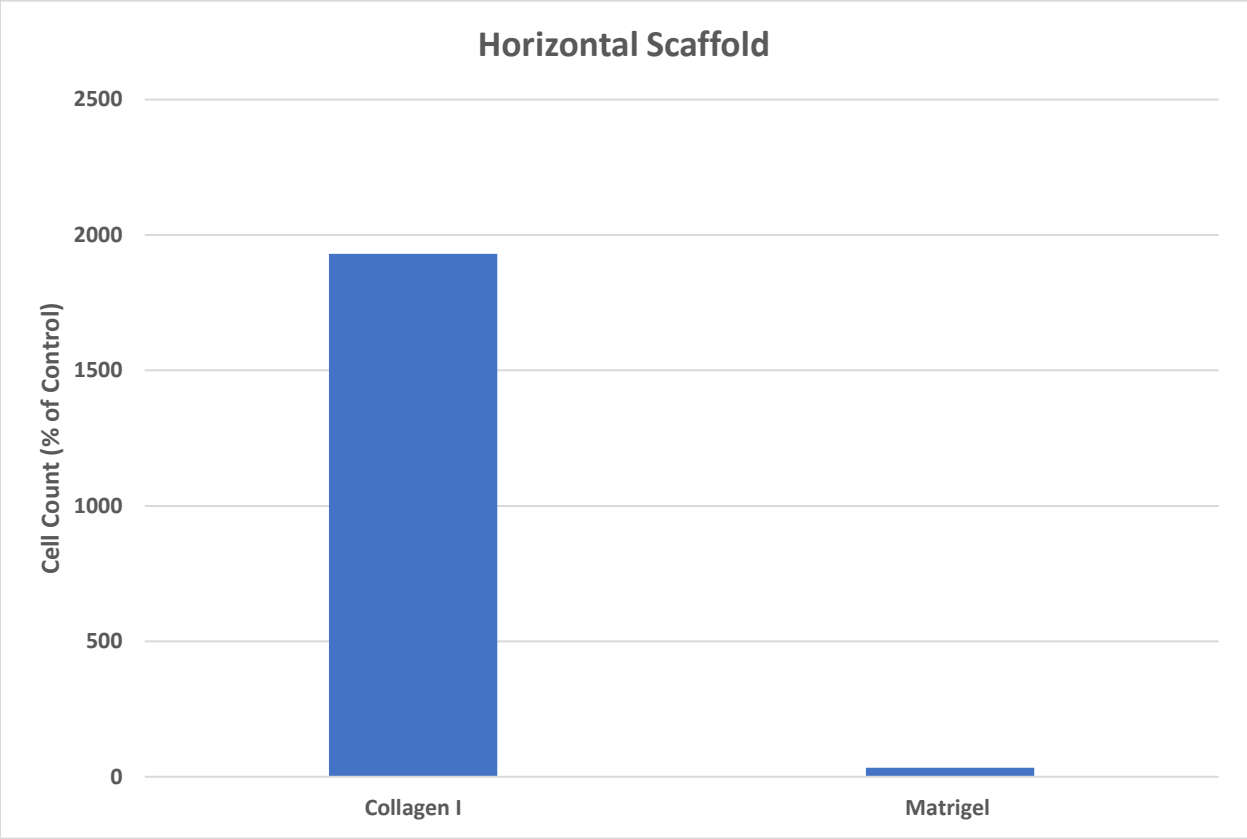


Figure 21: Horizontally Printed Scaffold Results (% of Control)

Total cells calculated from Table 3 were compared to the horizontal control group using a bar chart for each horizontally printed experimental condition, refer Figure 21. For the horizontally printed scaffolds, Collagen I provided extremely higher cell counts compared to the matrigel and control groups. Error bars are not provided as only one replicate was performed.

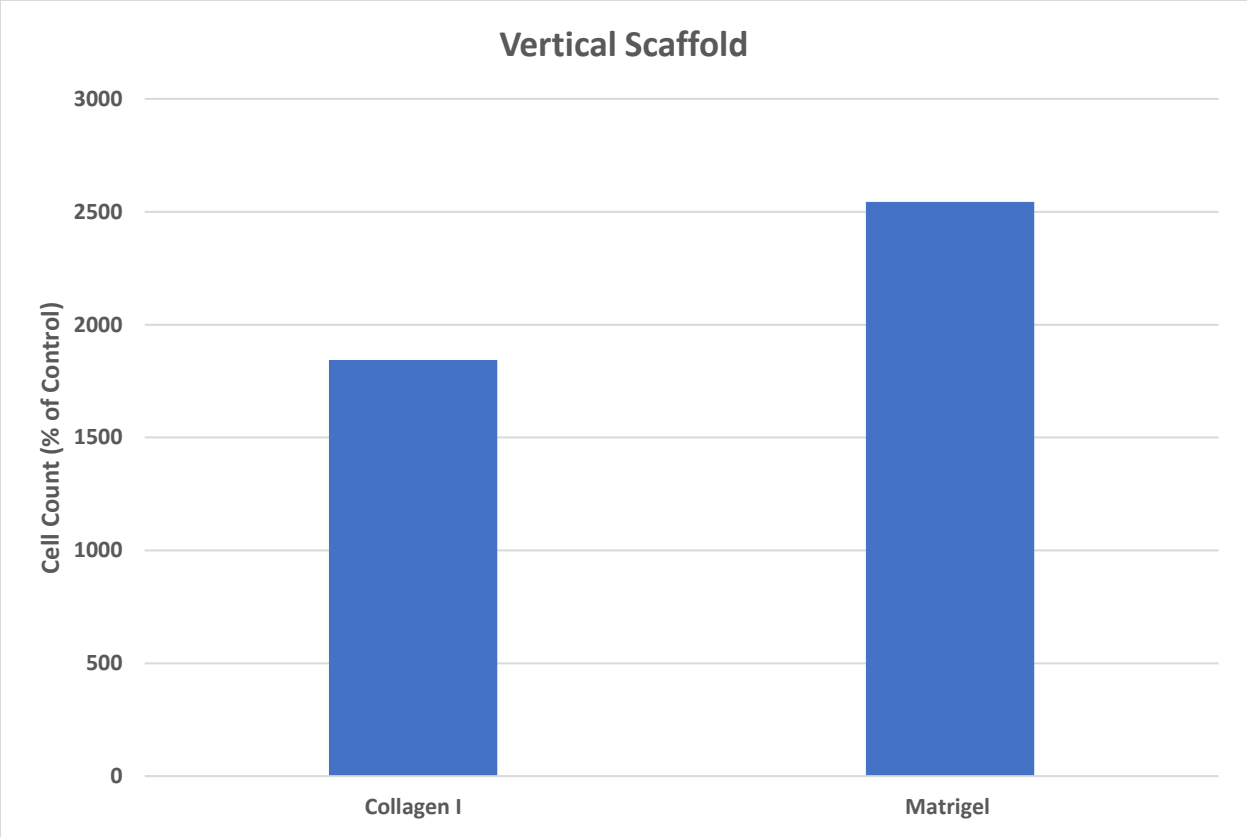


Figure 22: Vertically Printed Scaffold Results (% of Control)

Total cells calculated from Table 3 were compared to the vertical control group using a bar chart for each vertically printed experimental condition, refer to Figure 22. For the vertically printed scaffolds, Matrigel provided the highest cell counts compared to the Collagen and control groups. However, Collagen I provided similar results to the Matrigel coated scaffolds. Error bars are not provided as only one replicate was performed.

DISCUSSION AND FUTURE WORK

Experimental Objective One

From the results, each experimental objective was successfully completed. For experimental objective one, cells did in fact adhere to the uncoated acid-catalyzed Poly(lactic acid) scaffolds using both printing orientations. However, cellular adherence was very limited and very negligible compared to the seeding density of five million cells. After four days and taking doubling time into account this surface does not promote a surface suitable for cellular adherence.

Experimental Objective Two

For experimental objective two, after introducing a protein substrate like collagen I, and Matrigel, cellular adherence did increase by at least 1800% with the exception of the horizontally printed scaffold coated with matrigel. Printing orientation significantly affected cellular adherence especially with the vertically printed scaffolds. Both protein substrates promoted a framework that increased cellular adherence compared to the control scaffold. For the horizontal scaffold, collagen I increased cellular adherence compared to that of matrigel. From these results, it is not very clear why this is the case, especially when the cellular adherence in the vertical scaffolds were comparable. One factor could be that the concentration of matrigel was just too low. Or the size of the proteins that are in matrigel are just too large to adhere to the micro pore surface on the horizontal scaffolds. Matrigel consists of several different proteins, especially Laminen and Collagen IV. Laminen is a fairly large, folded protein that is approximately 400 to 900 kDa while Collagen IV is approximately 180 kDa. Laminen in particular folds into a very large protein structure. This may be one of the contributing factors to decreased cellular adherence.

Experimental Objective Three

For experimental objective cellular adherence was significantly increased in the vertically printed scaffolds. The outer most surface is very solid with a low void fraction. Protein substrates can coat the entirety of the surface and provide more surface area for cells to adhere to. But the void fraction is something that needs to be seriously considered as the horizontally printed scaffolds could provide cells with the ability to supply nutrients and dispose of cellular waste. From the results, horizontally printed scaffolds should be further researched using a collagen substrate.

Future Work

In order to statistically verify these finding, this procedure should be repeated at a minimum of three times. If these results are in fact repeatable, optimizing the protein substrate concentrations for collagen should be investigated using a horizontally printed scaffold. Another thing that should be adjusted in this protocol is to change the test performed during takedown. Instead of lifting cells from the scaffold then counting, it would be more effective if another method were used. I would recommend using a cellular proliferation assay provided from CyQUANT (Cat#: C7026). This highly sensitive fluorometric assay works by assessing cellular proliferation by dying nuclear DNA. Then the cells are lysed, and the DNA content is measured using a fluorometric plate reader. Implementing this assay would reduce any inconsistencies with lifting cells from the scaffold and performing cell counts. DNA content is relatively consistent within each cell, this would allow this to be proportional to cell counting. Following these studies, some toxicological testing should be done to investigate the toxicological effects of cellular adherence using Poly(lactic acid) as a surface. Once, toxicological effects are determined then primary human cells should be used.

CONCLUSIONS

Tissue engineering is still in its infancy in terms of biomedicine. Only a few procedures have been successfully performed (skin grafts, minor arteries, and a full trachea). However, several organs have also been successfully grown. These procedures are especially expensive and time consuming to perform. As more research is performed on tissue engineering using inexpensive polymers, this can dramatically reduce the overall cost. Another impact tissue engineering may provide is by using a patient's own cells to manufacture a fully functional organ this will dramatically decrease the need for organ and tissue donations all together. From the contributions of this research, cellular attachment is indeed possible on Poly(lactic acid) surfaces. Especially with a substrate coated surface. This may provide a very promising and inexpensive polymer that deserves future research in tissue engineering.

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APPENDIX

LAB NOTEBOOK

In this appendix, scanned copies of laboratory notebook pages used during this thesis are included.

Thesis Experiment

Chips were provided by Prabjihat Singh on 14 Feb 2020
↳ Supplied by Prabjihat in glass 60 mm petri dishes
↳ 3 horizontal and 3 vertical chips

Agar Solution

- 5 grams of Agar (Agar Powder purchased from Amazon.com, Now Real Food) was added to 500 mL of DI H_2O under ~ to make a 1% wt/v solution
- Solution was mixed / inverted several times until homogeneous
- Autoclaved using Liquid 30 setting (121°C) for 30 minutes.
- Solution was allowed to cool (Touchable by hands) before use.

Note: Chips were sterilized by soaking in 70% EtOH then under UV for 30 minutes each before coating chips in Substrate.

Collagen I, Rat Tail Coating

- Gibco (cat number: A10483-01, Lot number: 2103638, storage: 4°C, exp: 2020-07-07)

Desired Concentration = 50 $\mu g/mL$
Stock Conc = 3000 $\mu g/mL$

$$V_{collagen} = \frac{(50\ \mu g/mL)(2\ chips)(5\ mL/chip)}{3000\ \mu g/mL} = 0.1667\ mL = 166.7\ \mu L$$

$$V_{30\ mM,\ AA} = [(2\ chips)(5\ mL/chips)] - 0.1667\ mL = 9.833\ mL$$

- 5 mL of Collagen Soln was added to each dish (1 horz + 1 vert)
- Allowed to incubate @ RT for 1 hr
- Soln was aspirated, chips were then washed 3X w/ 5 mL PBS
- Chips were allowed to air dry before use.

Stan K Hsu
6 MAR 2020

Thesis Experiment

Cat (P/N): 354277
Lot: 354 277
Conc: 10 mg/mL

Desired Conc = 0.25 mg/L

$C_1V_1 = C_2V_2$

$(10\ mg/L)V_1 = (0.25\ mg/mL)(2\ chips \cdot 5\ mL/chip)$

$V_1 = 0.25\ mL$ matrigel $V_{media} = 10\ mL - 0.25\ mL$

- Matrigel was thawed @ 4°C overnight in ice bucket w/ ice.
- Solution was to an ice cold 50 mL conical containing 9.75 mL media
- 5 mL of matrigel was transferred to each dish
- Chips were allowed to incubate @ 37°C overnight.

- 20 uL of Agar was added to each (6) 100 mm petri dish
- The agar was allowed to set ~1 minute to solidify
- Chips were placed & pushed into Agar
- Agar and chips were allowed to set for 30 minutes @ RT to fully solidify.

6 mar 2020

Thesis Experiment

Cell Plating

- Media Needed = 6 plates × 5 mL/plate = 30 mL
- Cells Needed = 5(10^6) cells/plate × 6 plates = 30(10^6) cells

- HUVEC Cells
  P: 30
  Lot: 2015.05.29
  (Provided by IONTOX)
  ATCC
  Cat: CRL-1548

- Cells (① media) were aspirated from T-150 flask (CytoOne, cc7682-4825) using an aspirator. (Sept 6 mar 20)
- Cells were rinsed w/ 15 mL PBS (1X) then aspirated
- 4 mL of TrypLE (1X) (Life, Cat: 12563011) was added to flask and evenly spread.
- Flask was incubated ~10 min until cells lifted
- 9 mL of fresh media was added to flask
- entire suspension was transferred to 15 mL conical
- Centrifuged @ 200 G for 7 minutes
- Supernatant was removed via aspirator
- Cell pellet was resuspended in 10 mL fresh media

Counting Cells

- 25 µL of Trypan Blue (HyClone, SV30084.01) was transferred to a 1.5 mL eppendorf tube.
- 25 µL of resuspended cells was transferred to eppendorf.
- 10 µL of Cell/Trypan-blue was added to two Countess slides
- Cells were counted using automated Cell Counter (Countess II)

Count 1: 4.16(10^6) cells/mL
Count 2: 3.85(10^6) cells/mL

$$\text{Total Cells: } \frac{(4.16 + 3.85)}{2} \times (10^6)(10\text{ mL}) = 40\,050\,000 \text{ cells}$$

$$\text{Cell Suspension Needed} = \frac{30\,000\,000}{40\,050\,000} \times \cancel{10} \text{ (②)} \times 10\text{ mL} = 7.5\text{ mL} \quad \text{(Sept 6 mar 20)}$$

$$\text{Media Needed} = (6 \text{ plates} \times 5\text{ mL/plate}) - 7.5\text{ mL} = 22.5\text{ mL}$$

① media, not cells
② Not in equation, error

6 mar 2020

Thesis Experiment

- 7.5 uL of Cell suspension + 22.5 uL of Media was transferred to a 50 uL Conical
- Cells are mixed thoroughly by inversion
- 5 uL of Cells were transferred to each plate containing scaffold. Care was taken to seed cells only the Apical side of the scaffold and not Agar
- Plates were incubated for 1 hr to allow cells to attach.
- 15 uL of additional media was added to each plate

Note: Medium was replaced daily (20 uL)

Chip Dimensions: (35.4 x 35.4 x 2) mm
pore size ~ 100 x 100 μm

Note: Cells scaffolds started to lift from Agar on day 3.

9 mar 2020

Thesis Experiment

Tear Down Procedure

- Chips were transferred to a new petri dish
- 3 mL of TrypLE was added to each chip
- Using a 1 mL p-pette, the TrypLE was agitated onto the surface after 5 min incubation.
- 5 mL of medium was added to each chip to neutralize TrypLE.
- Chips were washed 3x w/ medium / TrypLE soln.
- Each soln was transferred to separate 15 mL centrifuge tubes.
- Each tube was centrifuged @ 200 rcf for 7 minutes (salt in medium)
- Supernatant was aspirated and pellets were resuspended in 5 mL medium
- Cells were counted using same protocol.

Horizontal Control

C1 = 0
C2 = $3.52(10^4)$ /mL
AVG = 88000

Horizontal Collagen

C1 = $3.34(10^5)$ /mL
C2 = $3.46(10^5)$ /mL
AVG = 1700000

Horizontal Matrigel

C1 = $5.26(10^3)$ /mL
C2 = $5.86(10^3)$ /mL
AVG = $5.86(10^3)$ /mL

Vertical Control

C1 = $5.86(10^3)$ /mL
C2 = $3.52(10^4)$ /mL
AVG = 102650

Vertical Collagen

C1 = $3.46(10^5)$ /mL
C2 = $4.11(10^5)$ /mL
AVG = 1892500

Vertical Matrigel

C1 = $4.69(10^5)$ /mL
C2 = $5.75(16^5)$ /mL
AVG = 2610000

① 8 minutes was used not 7.

V March 22

5