Manufacture and Validation of Advanced Cell Culture Inserts

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Manufacture and Validation of Advanced Cell Culture Inserts

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Abstract

In the field of biomedical research, there is a need for more biomimetic *in vitro* cell models that more closely replicate *in vivo* cell behavior in order to improve the reliability of advanced tissue models and experimental techniques such as high-throughput drug screening. The use of cell culture plate inserts with biodegradable membranes can help improve *in vitro* models by providing an environment that better replicates a cell's natural extracellular matrix. However, all cell types have different environmental conditions and cues. Therefore, membranes must be optimized to the desired cell type. We aim to manufacture membranes that promote the proliferation and differentiation of muscle cells. To determine an optimized membrane chemistry, we fabricated four electrospun nanofiber membranes and cultured C2C12 mouse myoblasts on the membranes to assess cell viability. The chemistries chosen were PLA 100, PLA/PGA 85/15, 1:1 PLA 100:PGA/PCL 75/25, and PGA/PCL 60/40. Spin conditions were optimized for each chemistry to produce a tunable fibrous structure within the membranes. After culturing C2C12 cells on the membranes for 48 hours, all membrane compositions were determined to be viable options for culturing muscle cells, with cell viabilities of 72.27 ± 23.41%, 76.68 ± 19.02%, 89.17 ± 7.19%, and 97.85 ± 3.72%, respectively. These results, however, don't identify one specific optimized membrane for myoblast cell culture compared to the others, meaning further experimentation is needed to identify optimal membrane characteristics.

Keywords: Electrospinning, Myoblasts, Cell Culture Inserts

Introduction

Background

Two-dimensional (2-D) cell culture methods are well established as a valuable tool for research in many disciplines including drug development, tissue engineering, and other clinical fields. Currently, most cell-based studies use traditional flat and rigid substrates to culture 2-D monolayers of cells. However, in recent years, several limitations of this method have been identified, including the lack of a fibrous extracellular network that cells can interact with and the lack of three-dimensional (3-D) growth [1]. It has been widely recognized that the extracellular matrix (ECM) plays a critical role in stem cell differentiation, tissue repair, cellular communication, and more [2]–[4]. The lack of these native characteristics may lead to inaccurate and misleading results when using cell culture research as a way to predict cell behavior and response *in vivo*. This is especially applicable to the fields of tissue engineering and drug development, as the cells being cultured are intended to recapitulate *in vivo* cell behavior, and therefore may be implanted into the body or used to test how cells within the body respond to treatment. The drug development process begins with 2-D cell culture studies to provide evidence of the drug's effectiveness, followed by animal trials and clinical human trials. The majority of drugs that meet Phase I success metrics fail in Phase II or III due to lack of efficacy [1]. For example, only about 10% of anti-cancer drugs make it past clinical development due to high toxicity levels or low efficacy [5]. These results are due, in part, to the lack of a physiologically relevant screening process in pre-clinical trials and 2-D cell culture studies. These drug screening trials are performed in environments that do not sufficiently mimic the *in vivo* environment and therefore, provide somewhat inaccurate data for

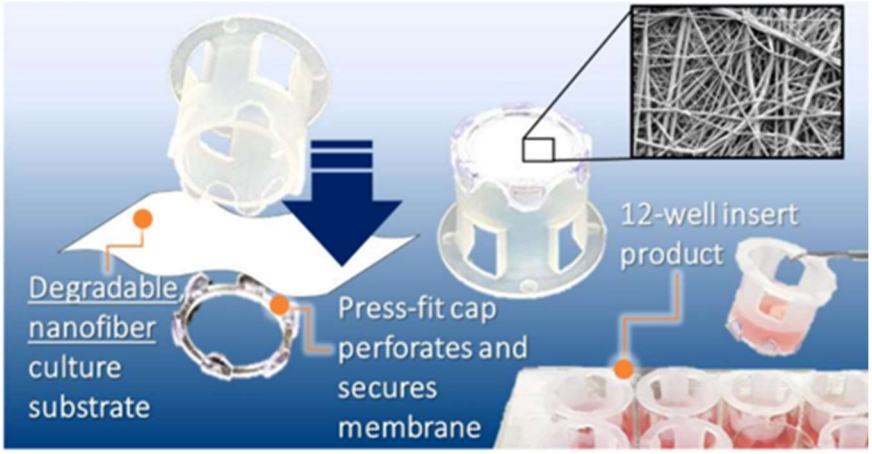

Figure 1: Luna Labs' RESORB membrane inserts featuring a degradable nanofiber membrane and a manufacturing focused design [7]

future applications of the drug. Billions of dollars are invested into the clinical development of drugs, which emphasizes the need to improve the screening process in the earliest stages of development. Over the past decade, much research has been dedicated to creating a more biomimetic cell culture environment [6]. This has led to the development of 3-D cell culturing scaffolds and structures. However, there are still many improvements to be made.

Previous Work

Luna Labs made progress on a solution to this problem through their RESORB cell culture inserts project, funded by an NIH contract [7], [8]. In Phase I of this project, they developed reusable plastic cell culture inserts for a 12-well plate that could be used to hold various biodegradable electrospun nanofiber membranes for cell culture purposes (Figure 1). These insets utilize snap-and-fit technology so that the sections of nanofiber membranes in the insert can be removed and interchanged, allowing for customization. Luna conducted a series of experiments and tests to optimize spinning protocols for different membrane chemistries and to characterize membrane properties such as fiber diameter, pore size, and degradability [7]. The goal of our capstone project was to build off of their research by manufacturing and validating these electrospun membranes to promote cell viability in C2C12 mouse myoblasts.

Aims

Aim 1. Our first aim was to design and manufacture an electrospun nanofiber membrane to act as a more biomimetic substrate for muscle cell culture. This was achieved by varying the polymer composition to create several membrane prototypes that mimic the ECM structure found in *in vivo* muscle cell growth.

Aim 2. Our second aim was to validate the membrane's functionality through *in vitro* cell culture. This was achieved by culturing C2C12 mouse myoblasts on the membrane prototypes and measuring cell viability using live/dead fluorescence staining and microscopy.

Specific Needs & Constraints

Design specifications for the inserts were identified based on literature for similar commercially available inserts and the constraints outlined in the NIH contract that funded Phase I of Luna Labs' research. Design specifications included high cell viability, sterilizability, biodegradability, and membrane thickness (Supplementary Table 1). In order to match standard cell culture plates, cell viability on the electrospun membranes needed to exceed at least 70%. This was verified using fluorescent live/dead staining. The membrane and insert needed to withstand ethylene oxide sterilization, which was the sterilization method previously used by Luna Labs during the development of the RESORB inserts. This was verified by qualitatively assessing fiber formation before and after sterilization using a light microscope. Because

membrane degradation was a critical design element that contributed to the novelty of Luna Labs' design, membranes needed to be capable of degrading at variable rates over 0-6 weeks of culture. Information regarding the degradability of different chemistries was obtained from discussions held with Luna Labs, as they had previously conducted chemistry degradation experiments and observational studies during Phase 1 of the RESORB project. Lastly, ideal membrane thickness was defined in Luna Labs' contract solicitation as less than 10 μm; although, less than 12 μm was considered acceptable for this project [8]. This specification was verified by measuring the membrane thicknesses using a micrometer.

Methods/Materials

Aim 1: Manufacture (Electrospinning)

An Elmarco Nanospider electrospinning machine was used to fabricate the nanofiber membranes for cell culture. The first step in the protocol was determining the chemical compositions of the membranes to be tested. A literature review was used to assess the degradation rate and compatibility of polymers and polymer combinations [9], [10]. The selected chemistries were dependent on the available inventory provided by Luna Labs and their prior work. The available polymers were researched and the four compositions chosen for comparison were PLA 100, PLA/PGA 85/15, 1:1 PLA 100:PGA/PCL 75/25, and PGA/PCL 60/40 in order of slowest to fastest degradation rate.

The protocols for spinning specific solutions were determined with guidance from advisors at Luna Labs. For each experimental spin, the following conditions were adjustable using the touch screen interface of the Nanospider: carriage speed, orifice diameter, applied voltage, and substrate speed. Luna Labs provided their records of spin conditions and how they were optimized for these chemistries so that they could be referenced when selecting spin conditions. However, this information is not included in the report as it is proprietary.

After determining the optimal spinning conditions, the membranes were spun for experimentation. The substrate and membrane were removed from the Nanospider and the membrane's thickness was qualitatively assessed. The region on the substrate that would produce the most durable and uniform membranes was identified. Membrane thickness was then quantitatively characterized in this region using a micrometer. Nine measurements of thickness were taken for each chemistry's selected membrane region and averaged.The sample was then imaged using a light microscope to confirm the presence of a consistent fibrous structure.

The inserts were used to perforate the membrane by pressing the snap-fit cap onto the insert with the membrane pulled taut between. Excess membrane was trimmed from around the insert. Plasma treatment, a process that increases the hydrophilicity of the membrane surface, was then performed on all inserts, except for 12 PLA 100 inserts which were used as the non-plasma treated condition for the first validation experiment. Plasma treatment allows for better hydration of the membrane, promoting cellular adherence [11]. Inserts were placed in a Harrick Plasma Basic Plasma Cleaner. The plasma cleaner was then turned on for 3 minutes in order to treat the membranes. Then all inserts, plasma treated and non-plasma treated, were sterilized using ethylene oxide sterilization. These sterilized inserts were then ready for experimentation to validate the membranes of various chemistries.

Aim 2: Validation (Cell Culturing)

To validate functionality, the membranes were seeded with the immortal mouse myoblast C2C12 cell line. C2C12 cells would reveal if the properties of optimized membranes promoted muscle cell proliferation. This cell line was also chosen due to its rapid cellular division rate [12]. Rapid proliferation allows for cellular adherence and confluency to happen quickly, limiting experimental timeframes to two days. This also allowed for high turnover rates between iterations of experimentation.

All experiments were conducted in 12-well plates. A sample size of 3 wells was used for each condition of the experiments. C2C12 cells at a confluence level of 70-90% were harvested. These cells were then counted and resuspended in cell growth media at a cell density of 0.1 million cells/mL. The growth media consisted of high glucose + glutamine (no sodium pyruvate) Dulbecco's modified eagle medium (DMEM)

#11965, fetal bovine serum (FBS) (10%) and antimicrobial antibiotic (AA) (1%). 1 mL of cell suspension was added to each experimental well. Cells were then cultured in an incubator (37 ℃, 95% humidity, and 5% CO_2) for 48 hours.

After 48 hours in culture, the cells were prepped for staining and imaging. The experiment plates were checked under a light microscope to ensure cellular adherence in the control wells. Staining solutions were then made by adding 6 drops of NucBlue and Propidium Iodide each to 4 mL of growth media to make the live/dead stain, and by adding 6 drops of Phalloidin to 4 mL of growth media to make the actin stain. The stain solution tubes were wrapped in aluminum foil to limit the exposure of the stains to light. Working with the wells of one experimental condition at a time, the growth media was aspirated and the wells were washed twice with 1 mL of phosphate-buffered saline (PBS). 200 µL of live/dead stain was then added to each well/insert. The stained membrane, or plate well of the control condition, was then incubated in a dark drawer at room temperature for 5 minutes. For a given membrane condition, the three membranes were removed from the inserts and placed, cell side down, on a microscope slide adjacent to each other. Each membrane or plate well was then imaged using a Life Technologies fluorescent light microscope at 20X magnification. In-focus brightfield, RFP channel, DAPI channel, and RFP/DAPI overlay images were taken at a single field of view for each sample. 200 µL of actin stain was then added to the membranes or wells, incubated for 10 minutes, and imaged again, in order to obtain an RFP/DAPI/GFP overlay image for each sample. This process was repeated for each of the other conditions in the experiment.

The cell viability was then determined for each experimental condition. The DAPI channel images were used to manually count the total number of cells for each sample. The RFP channel images were used to manually count the number of dead cells for each sample. Using these counts, the cell viability percentage of the samples was calculated using the following equation:

$$cell\ viability = \frac{total\ cells - dead\ cells}{total\ cells} \times 100 \quad (1)$$

The 3 sample cell viabilities, found from Equation 1, for each condition were averaged and the standard deviation was found.

Two validation experiments were performed. The first experiment was the plasma treatment trial, which included a standard well control condition, plasma treated PLA 100 condition, and non-plasma treated PLA 100 condition. This experiment was performed to determine the effect of plasma treatment on cellular adherence and cell viability. The second experiment was the membrane composition comparison trial, which included a standard well control condition and four different membrane chemistries: PLA 100, PLA/PGA 85/15, 1:1 PLA 100:PGA/PCL 75/25, and PGA/PCL 60/40. All membranes were plasma treated in this experiment. This experiment was performed in order to determine the optimal membrane composition for muscle cell growth.

Results

Membrane Characterization

After spinning, all membrane chemistries were imaged to assess fiber formation. As seen in

Table 1. Membrane Thickness Measurements

Membrane	avg. thickness ± 1 std. dev. (µm)
PLA 100	7.74 ± 2.81
PLA/PGA 85/15	6.75 ± 2.19
1:1 PLA 100 : PGA/PCL 75/25	9 ± 4.10
PGA/PCL 60/40	6.33 ± 2.50

Figure 2, successful fiber formation was qualitatively observed for all experimental chemistries. Measurements of membrane thickness were also recorded for each membrane (Table 1). The PLA 100 chemistry had an average thickness of 7.74 ± 2.81 µm. The PLA/PGA 85/15 chemistry had an average thickness of 6.75 ± 2.19 µm. The 1:1 PLA 100:PGA/PCL 75/25 chemistry had an average thickness of 9.00 ± 4.10 µm. Lastly, the PGA/PCL 60/40 chemistry had an average thickness of 6.33 ± 2.50 µm. All of these measurements were below the ideal 10 µm maximum determined by the design specifications.

Plasma Treatment Experiment

Cell viability was recorded for the plasma treated PLA 100 membrane group, the non-plasma treated PLA 100 membrane group, and the control group when assessing the effects of plasma treatment on cell viability (Figure 3A). The plasma treated group had an average cell viability of 94.77 ± 3.21%. The non-plasma treated group and control group had an average cell viability of 96.05 ± 2.68% and 100 ± 0%, respectively. A one-tailed ANOVA test was performed with a p-value of 0.085, indicating that none of the experimental groups were statistically significantly different from one another.

Chemistry Comparison Experiment

Cell viability was recorded for the PLA 100 chemistry group, the PLA/PGA 85/15 chemistry group, the 1:1 PLA 100:PGA/PCL 75/25 chemistry group, the PGA/PCL 60/40 chemistry group, and the control group to assess the effects of membrane composition on cell viability (Figure 3B). The PLA 100 chemistry and the PLA/PGA 85/15 chemistry had the lowest average cell viabilities of 72.27 ± 23.41% and 76.68 ± 19.02%, respectively. The control group had a cell viability of 87.54 ± 6.44%. The PGA/PCL 60/40 chemistry and the 1:1 PLA 100:PGA/PCL 75/25 chemistry had average cell viabilities of 97.85 ± 3.72% and 89.17 ± 7.19%. A one-tailed ANOVA test was performed with a p-value of 0.262, indicating that none of the experimental groups were statistically significantly different from one another. All of the chemistries had cell viabilities above the 70% threshold outlined in the design specifications.

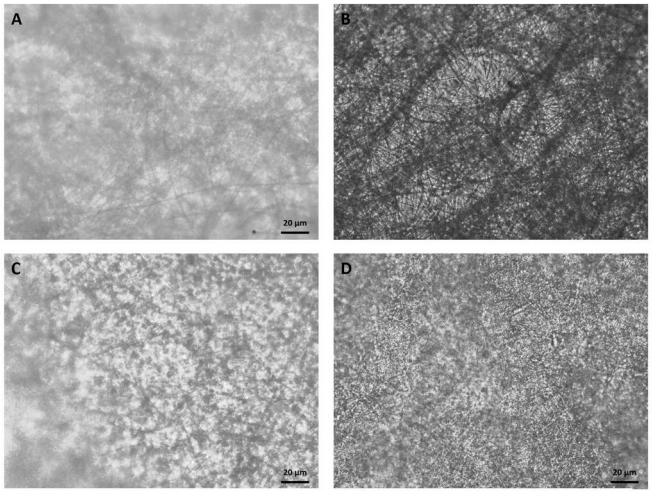

Figure 2: Light microscope membrane images, 40x magnification. A: PLA 100, B: PLA/PGA 85/15, C: 1:1 PLA 100 : PGA/PCL 75/25, D: PGA/PCL 60/40

Discussion

Explanation of Results

When comparing the effects of plasma treatment on cell viability, no statistical differences between the three treatment groups (plasma treated, non-plasma treated, and well plate control) were observed. Qualitatively, the plasma treated membranes and control wells appeared to have a more uniform distribution of cells in comparison to the less homogenous nature of the cells on the non-plasma treated membranes, as evidenced by the clustering of cells (Supplemental Figure 1). These findings suggest that the increased hydrophilicity of plasma treated membranes promotes uniform cell distribution. While more research is required to fully understand the effects this may have on cell development, uniform distribution of cells may support more efficient proliferation and differentiation into the target cell populations. Homogenous distribution may additionally promote more consistent tissue formation, ECM deposition, and membrane degradation, further aiding in the potential for these inserts to promote biomimetic cellular growth. As previously mentioned, cell to cell interactions play a large role in cellular behavior, and the clumping of cells could potentially have negative effects on the ability of cells to behave as they would *in vivo* [13]. As expected, qualitative observation showed

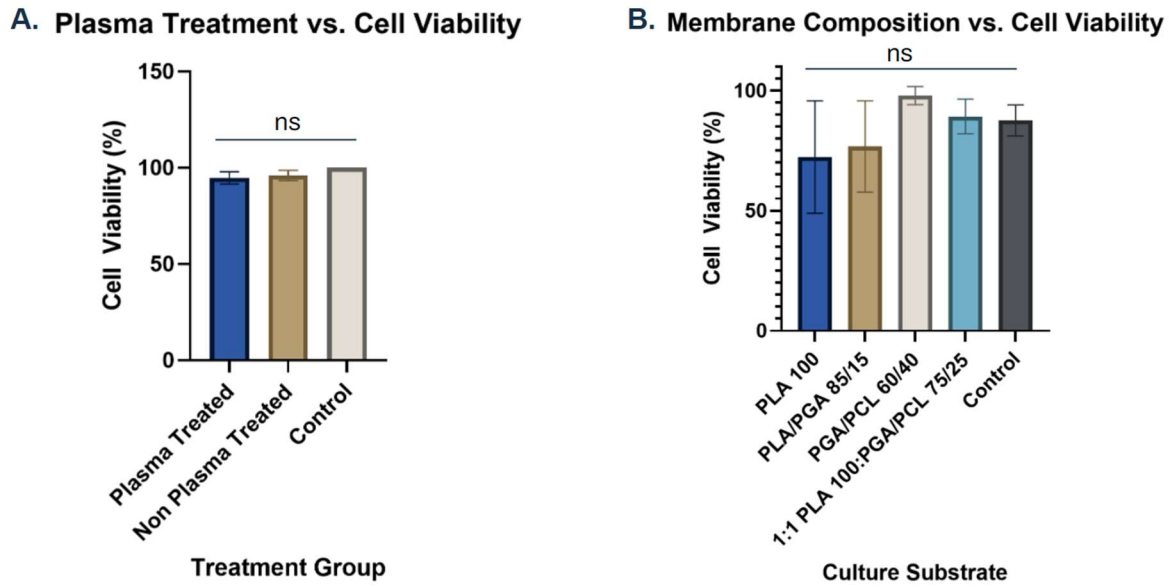

Figure 3: Average cell viability (± 1 std. dev.) for (A) Plasma treatment experiment (p value = 0.085) and (B) membrane composition experiment (p value = 0.262). ns = not statistically significant through one-tailed ANOVA test

that the plasma treated membranes hydrated faster than non-plasma treated membranes. Although Luna Labs did not study the effects of plasma treatment on cell viability, their assessment of the effects of plasma treatment found that it increased hydrophilicity and membrane permeability [7]. Based on this data and the qualitative results previously mentioned, all membranes used in subsequent experiments were plasma treated, including the chemical composition comparison experiment.

The results of the chemical composition comparison experiment were also not statistically significant. Because of this, an optimal membrane chemistry could not be definitively concluded. While non-significant, there was an apparent increase in cell viabilities for the 1:1 PLA 100:PGA/PCL 75:25 and PGA/PCL 60:40 chemistries, which were the most degradable membranes. Further experimentation with a larger sample size is required to determine if this increase is significant. Limitations in imaging may also have affected some of the cell viability data. It was more difficult to focus on a single plane of cells when imaging the 1:1 PLA 100:PGA/PCL 75:25 and PGA/PCL 60:40 membranes (Supplemental Figure 2). This is likely due to the uneven surface of the membrane and the ability of cells to penetrate into the fibrous structure. While these characteristics were actually desired, as they more realistically mimic *in vivo* growth compared to a 2-D monolayer, it does affect the quality of imaging.

Although there wasn't a clearly superior chemistry in these initial studies, all membranes met the benchmark of 70% cell viability as defined by the design specifications. Additionally, all four membranes met the specifications outlined during the design process, including withstanding ethylene oxide sterilization, possessing various levels of biodegradability, and achieving a membrane thickness of less than 10 μm. This demonstrates that all the membranes explored are viable candidates for future cell culture experimentation and validation.

Future Directions

To better characterize the insert membranes and determine whether there is a significant difference between chemistries, future studies would benefit from more trials and further membrane optimization. This includes performing experiments with larger sample sizes to reduce the effects of biological variation between samples. Future research may also include the use of human cell lines in duplicated versions of the trials run to further demonstrate validity and applicability to human tissues and various cell types. Human cells

are often more sensitive than immortal cell lines like the C2C12 cell line and could potentially react to the membranes in different ways. To determine the effects of biodegradation on cell proliferation and viability and how it may influence natural ECM protein production by the cells, longer-duration experiments would also be beneficial. Additionally, more work can be conducted to evaluate membrane compositions, such as examining fiber alignment and fiber density or thickness, and evaluating the effects these characteristics may have on cell differentiation. The tunability of the membranes would allow for potential alterations to be made as membranes are customized for specific cell types.

As previously mentioned, some limitations were experienced during the imaging protocol. Other forms of quantitative analysis could also be performed to more accurately and precisely characterize cell viability. For example, cell viability could also be measured using a lactate dehydrogenase (LDH) assay and a colorimetric plate reader, which would remove the need for fluorescence microscopy. These additional studies would allow for further validation and identification of an optimal membrane for muscle cell growth. Future work contributes toward the goal of commercializing the inserts and membranes, so they can be used for tissue engineering research and high-throughput drug screening in a variety of labs.

End Matter

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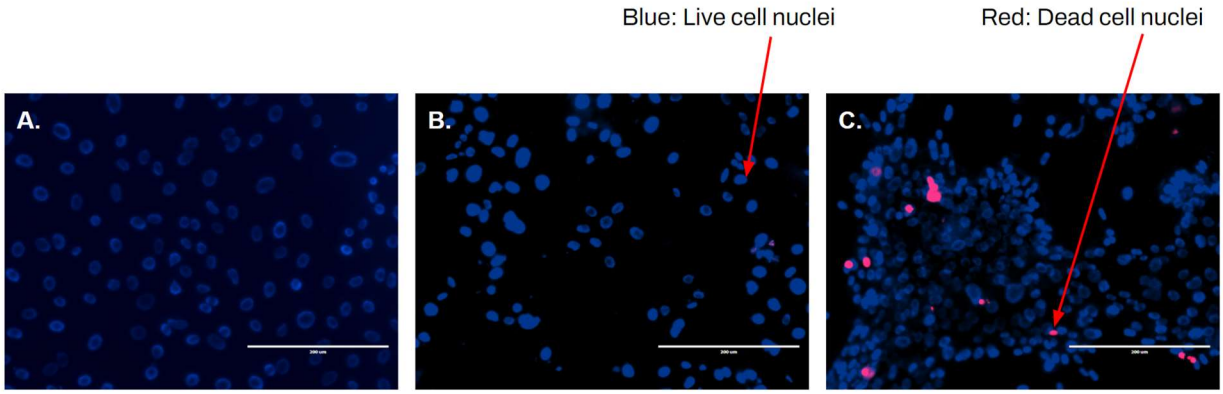
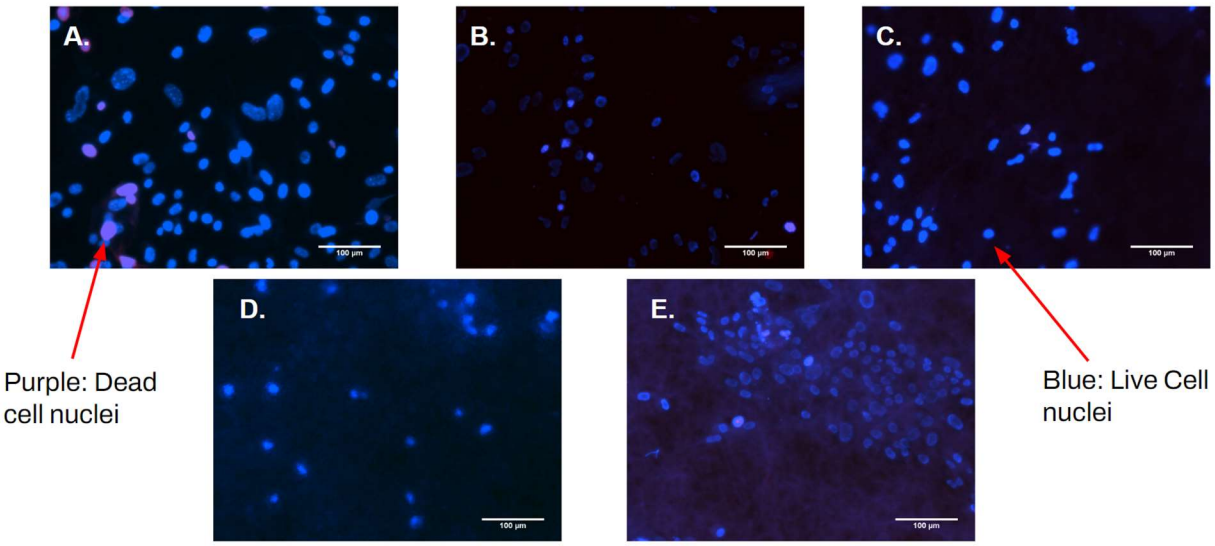
Supplemental Material

Supplementary Table 1. Design Specifications

Design Constraint	Unit of Measure	Marginal Value	Ideal Value
Cell Viability	Percentage of live cells (%)	> 70%	100%
Sterilizable	Withstand Ethylene Oxide Sterilization	Withstands Sterilization	Withstands Sterilization
Biodegradable	Weeks	0-6 weeks	0-6 weeks
Membrane Thickness	Micrometers (μm)	< 12 μm	< 10 μm



Supplemental Figure 1: C2C12 cells stained with NucBlue and Propidium Iodide. A. Control well B. Plasma treated PLA 100 membrane C. Non-plasma treated PLA 100 membrane. Images taken at 20x



Supplemental Figure 2: C2C12 cells stained with NucBlue and Propidium Iodide. A. Control well B. PLA 100 membrane C. PLA/PGA 85/15 D. PGA/PCL 60/40 E. 1:1 PLA 100:PGA/PCL 75/25. Images taken at 20x