Electrospun Poly-4-Hydroxybutyrate for Hernia Repair

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Technical Project Team Members

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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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Abstract

Hernias are a common occurrence, with over one million hernia repairs performed each year in the United States[1]. The current standard-of-care knitted mesh used in hernia surgical repair today leads to 6% of patients having long-term pain five to ten years following surgery[2]. Our team, in conjunction with Dr. George Christ at the Biomedical Engineering Department of the University of Virginia and Becton, Dickinson, and Co., worked to complete initial feasibility testing of an electrospun hernia mesh. Unaligned electrospun samples were hand-stretched to mimic aligned fibers in order to compare cell viability of C2C12 mouse myocytes among unstretched (0% strain), 20% strain, 50% strain, and control. Overall, we developed a cell culture protocol for seeding myocytes on the electrospun material to ensure reliable and reproducible results, though we found that the cell viability was very low across all samples when compared to control. Morphology was not able to be quantified due to the low cell viability. While these initial results were not positive, the stretching and seeding protocols combined with the findings on the current version of the hernia mesh will drive future research towards the development of a successful electrospun P4HB hernia mesh.

Keywords: Poly-4-Hydroxybutyrate, Hernia Repair, Electrospinning, Cell Viability

Introduction

A hernia occurs when an internal organ or body part pushes through a weak spot in muscle or tissue. The main types of hernias include inguinal, femoral, umbilical, and hiatal, with inguinal and femoral being the most common. Hernias can be caused by weakened muscles and/or excessive strain. Common types of strain result from pregnancy, physical exertion, obesity, frequent coughing, and bowel movements. On the surface of the body, a hernia looks like a bulge or lump that can be pushed back in and may disappear when the patient lies down[3].

More than 20 million inguinal hernia repair surgeries are performed worldwide annually[3], a surgeon pushes the internal organ or body part back in and reinforces the muscle or tissue wall with hernia mesh. These repairs are absolutely necessary, as patients will not recover naturally over time from a hernia. If left untreated, hernias can lead to obstruction of the intestine or strangulation, preventing ample blood supply from reaching necessary areas of the body. The cost associated with surgical hernia repair usually totals around $3500 - $4500[4]. On top of the expensive surgery, many patients do not make a full recovery in the weeks, months, and even years after surgery. Despite multiple options and advances in surgical hernia repair, 10-12% of patients suffer from chronic pain following their surgery and 11% of patients suffer from a recurrence[5]. With each recurrence comes added costs, increased risk of infection and re-recurrence, as well as specific complications like testicular atrophy.

Hernia mesh has evolved over the last 50 years, each mesh coming with pros and cons; there is no perfect mesh currently on the market[4]. The quality of life of hernia patients often takes a serious hit due to muscle trauma from atrophy, injury, or the surgical procedure. Poly-4-hydroxybutyrate (P4HB) knitted Phasix™ mesh has been one popular biomaterial used for hernia repair, though there are still issues present with muscle regeneration in these patients[6]. P4HB is a naturally derived and fully absorbable polymer produced by E. Coli K12 bacteria by means of transgenic fermentation techniques. P4HB degrades *in vivo* through hydrolysis and a hydrolytic enzymatic digestive process and is fully resorbed in approximately 12 to 18 months[7]. The material has proved to be advantageous as a biomaterial because of its ability to provide both consistent support and a long-term resorption profile to prevent hernia recurrence. P4HB

currently exists as a knitted mesh where the scaffold becomes overlaid with non-functional scar tissue[8]. However, an electrospun scaffold has the potential for infiltration and regeneration by aligned myocytes to aid in functional repair of tissue[9]. Functionally designed biomaterials hold the potential to recruit resident cells and recapitulate features of the native ECM[10]. We believe that looking at electrospun P4HB mesh, rather than knitted mesh, may be beneficial, as the method of electrospinning lends itself to tunable fiber alignment, porosity, and fiber thickness.

P4HB has great utility as a hernia mesh biomaterial due to its long-term strength profile and its ability to be well-received by the body. P4HB repairs demonstrate a consistent strength profile with repair strengths significantly greater than the native abdominal wall over time, despite resorption of the P4HB[7]. Additionally, P4HB's slow degradation in the host allows for a gradual change in mechanical properties and transferring of loads from the mesh scaffold back to the tissue[11]. P4HB has also been found to simulate a higher M2:M1 macrophage cell ratio compared to other meshes[12], eliciting a pro-regenerative, anti-inflammatory response. This is advantageous because most complications following hernia repair surgery are associated with inflammation at the implant site.

Materials and Methods

Electrospinning

Electrospinning is a technique to produce a variety of polymeric nanofibers; the scaffolds have the potential to facilitate improved wound healing postoperatively by controlling the organization of regenerating tissues[13]. The project seeks to combine two proven technologies in P4HB material and electrospun scaffold to create a hernia mesh that is safe and resorbable, but also provides functional tissue regeneration. Electrospun scaffolds were sent to the Dr. Christ lab for testing in a sterile package.

Stretching Protocol

Under the laboratory hood, three 2.5 cm x 2.5 cm samples were cut from a larger sterilized sample of size 4 in x 4 in. The 0% strain sample was placed directly into a 5 cm well plate and covered. The 20% strain sample was stretched in the direction of relative fiber alignment gently once every five seconds 12 times until the correct strain was achieved. The 50% strain sample was stretched in the direction of relative fiber alignment gently once every five seconds 17 times until the correct strain was achieved. The 50% strain value was chosen as the maximum because at 60-70% strain for a 2.5 cm x 2.5 cm piece of material, it becomes too thin and begins to break. 20% strain was chosen as a third data point between 0% strain and 50% strain to determine if a smaller amount of strain than 50% made a statistically significant difference in alignment. These strains were pre-calculated as a distance and measured with a ruler. Directly following stretching, the material was placed in a 5 cm well plate, covered with its respective top, and labeled. To ensure sterility following cutting pieces of the sample off for testing, the rest of the material was placed back in the paper envelope and further into the package the material was sterilized in. It was then taped airtight shut. The package was sprayed thoroughly with isopropyl alcohol each time it was taken out for additional sample cutting. Each sample (0% strain, 20% strain, 50% strain) sent to Becton Dickinson (BD) in Durham, North Carolina was placed in a 5 cm well plate, taped shut, and labeled. In Durham, BD took SEM images from the middle of the samples where the fibers were most aligned following the hand-stretching. The set up for the scanning electron microscopy can be seen in the figure below, sent by BD. The samples used for SEM imaging were destroyed following imaging and new samples were cut and stretched following the same protocol for use in the cell culture.

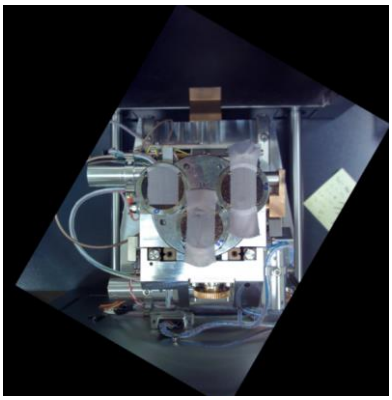

Figure 1. Electron Microscope Imaging Set-Up at Becton Dickinson in Durham, NC.

Stretching Quantification Protocol

SEM images were received back from the Becton Dickinson team. The highest magnified image from each sample (0% strain, 20% strain, 50% strain) was imported into ImageJ for analysis. The angle tool was used to measure the angle of 20 fibers from the center line. The averages and standard deviations were calculated to utilize in an ANOVA test to determine if there was a statistically significant difference in alignment of the fibers.

Seeding Protocol
After seeding, the material had to be held down. While the material is hydrophilic and absorbs water well, it still floats in the cell media. The samples were held down to ensure that cells attached rather than floated to the bottom of the plate and attached there instead. After testing several different methods, it was decided that small stainless-steel washers pre-sterilized in isopropyl alcohol were best suited to hold the material down over several days in media (see Figure 2 below). The washers were small enough to not cover the material completely, heavy enough to hold it down after days in the media, and were the easiest material to sterilize prior to placement on the material. In the long-run for testing this material, a 3D printed, customized seeding chamber would be the most reproducible and reliable method for seeding. After the washers were placed in each corner on the dry sample, 1 mL of C2C12 media was gently pipetted onto the material until the sample was saturated with media. The media-soaked samples were left to sit in the hood for five minutes before cell seeding.

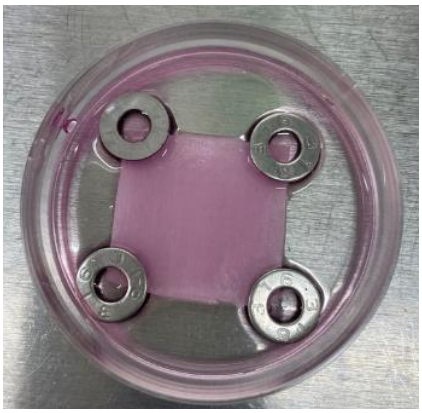

Figure 2: Final Cell Culture Design prior to seeding. *Four metal washers were used to hold the corners of the samples down to the bottom of the 5 cm well plate.*

While the samples were left under the hood to soak in media for five minutes, the cell suspension was prepared. A 10 cm plate of C2C12 of about 90% confluency was harvested. A typical cell harvesting procedure was utilized, where the media was gently aspirated and a 5 mL PBS wash was used. 3 mL of trypsin was pipetted into the well before the well plate was placed back in the incubator for 5 minutes to allow the trypsin to properly aid the cells in detaching from the bottom of the plate. This solution was collected in a centrifuge tube. A 5 mL PBS wash was used to collect any cells that still remain on the plate. The collected cell solution was centrifuged at 2,500 revolutions per minute for 5 minutes. The liquid was then aspirated and the pellet of cells was left at the bottom. 5 mL of media was deposited into the centrifuge tube and thoroughly mixed to separate the pellet. 1 mL of the cell slurry was removed for counting purposes. Following counting, one million cells were carefully deposited onto each sample based on the cell counting calculations. The well plates were then placed into the incubator with 5% CO_2 and 20% O_2.

Cell Quantification Protocol
The samples were incubated for t=48 hours following seeding. A staining solution was mixed together, containing 1 mL of C2C12 growth media, 1 drop of Propidium Iodide, and 1 drop of DAPI from the ThermoFisher Scientific Labeling and Detection Molecular Probes. After the samples were removed from the incubator, the media was carefully aspirated from the well and the metal washers were removed using forceps. 200 microliters of the media-dye solution was pipetted directly onto the sample in the well and the well plates were gently tipped to ensure that the media-dye solution coated the cells on the plate for the control. The samples were removed from the plates and placed on glass slides with a cover slip on top. Immediately following the plating, the samples were taken to a neighboring lab for EVOS fluorescence microscopy imaging. Each sample was placed under the microscope and moved around to find a high density of cells. The control of the representative pictures were taken on the red and blue setting separately for cell viability counting, with an additional overlay image for a more complete picture. For the purposes of this project, the control group was the cell and media mixture present in the same plate as the material but not in direct contact with said material. For cell counting, the blue pictures were used to count the total number of cells stained by DAPI in the field, and the red cells stained by Propidium Iodide were used to count the number of dead cells. The viability was calculated by the following equation from the values collected: (total cells - dead cells)/total cells.

Results

Stretching Results
Following the stretching protocol, the SEM images displayed in Figure 3 were received from BD. As stated

above, the ImageJ angle tool was used to measure the angle from 0° for 20 fibers. The average and standard deviation calculations are as follows: 0% strain: 45.02° ± 23.9°, 20% strain: 28.48° ± 17.1°, 50% strain: 16.6° ± 10.4°.

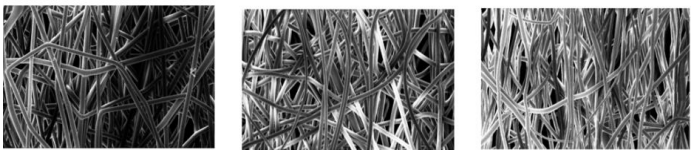

Figure 3: Electron Images of P4HB Fibers. *Images were taken by BD in Durham, NC and fiber alignment was quantified utilizing ImageJ.*

An ANOVA test was performed to determine if there was a statistically significant difference in alignment among the three groups. The results of the ANOVA test present in Table 1 indicated that there was a statistically significant difference.

Source	SS	df	MS		
Between-treatments	8171.5793	2	4085.7896	*F = 12.61168*	
Within-treatments	18466.2226	57	323.9688		
Total	26637.8019	59			
The f-ratio value is 12.61168. The p-value is .000029. The result is significant at p < .05.					

Table 1: ANOVA Analysis Results. *A statistically significant difference was found among the 0% strain, 20% strain, and 50% strain groups.*

Cell Quantification Results

As stated in the Materials and Methods section, an EVOS microscope was used to fluorescently image the samples. The representative overlaid images of Figure 4 suggest that there are very few cells (blue) in images A, B, and C when compared to control image D. Images from separate channels for only blue, only red, and an overlay image are featured in Appendix Figure 1. The quantification of these images for cell viability resulted in the following: 0% strain: 2.38%, 20% strain: 4.50%, 50% strain: 2.04%, control: 98%.

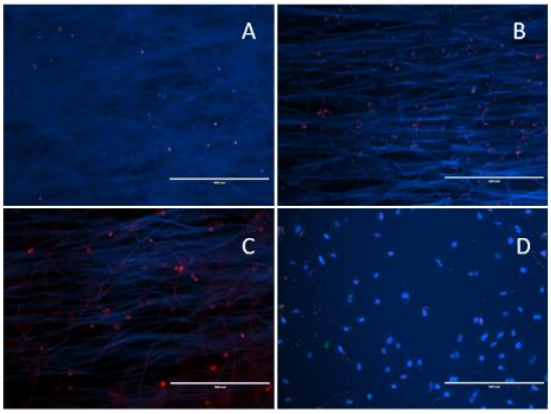

Figure 4. Immunofluorescence imaging displays low cell viability when compared to control. DAPI and Propidium Iodide stains on samples at 0% strain (A), 20% strain (B), and 50% strain (C) as well as control (D).

Discussion

The C2C12 cells present on the electrospun P4HB mesh displayed very low viability, regardless of what degree of strain the material was stretched to. Lower cell densities on the material than were originally plated were repeatedly observed during testing. These findings contradict the original hypothesis that aligned electrospun fibers would promote even more cell growth than a traditional scaffold.

These shortcomings can likely be attributed to an unfavorable reaction between the C2C12 cells and the material. C2C12 was selected as the cell line for the design project because it is highly resilient and capable of being cultured under a variety of conditions[14]. Because electrospun P4HB is a novel hernia mesh, there is limited research available on it and whether it has an affinity for the C2C12 cell line. However, BD has a knitted P4HB hernia repair mesh that has been cleared for clinical use which suggests the low viability could be the result of the conformational change induced by electrospinning the P4HB.

Another explanation for the low cell viability is that the material was not functionalized. Functionalization is a method of tailoring a biomaterial's surface in accordance with the physiological surroundings of the living cells[15]. Functionalization has been shown to produce a bioactive surface on biopolymers while preserving the bulk properties of said materials[16]. Both Lee et al., 2013[17] and Zhao et al., 2013[18] developed methods to functionalize biopolymer polyetheretherketone (PEEK) by means of

electron beam deposition and sulfonation, respectively. Biopolymers have lower heat resistance than other biomaterials which presents challenges during functionalization, by nature a heat intensive process, but these studies have laid the groundwork for further functionalization of biopolymers such as P4HB. Electrospun P4HB scaffolds are in their very early stages but going forward the material could be functionalized to present proteins and peptide sequences that encourage C2C12 cellular adhesion.

Limitations

The main limitations with the project stem from the lack of knowledge about the interaction between the material and the C2C12 cell line. Additionally, C2C12 mouse myocyte cells are not entirely indicative of how human myocyte cells will perform when exposed to the material.

Implications for Future Studies

While the current iteration of the hernia mesh needs work, through our project we discovered that C2C12 cells display an unfavorable reaction with a non-functionalized version of the material while simultaneously developing a successful stretching and cell culture protocol suited for testing future versions of the mesh. Future work on this project should explore how the electrospun P4HB material can be enhanced to induce a better reaction between the C2C12 cells and the material. We recommend that BD functionalize the next version of the electrospun mesh as well as run their knitted Phasix mesh through the developed protocol to determine whether the low viability is solely because of the electrospinning conformational change or an unfavorable reaction between the P4HB and C2C12 cells. Once the proper interaction between the material and C2C12 cells has been established, the study should then investigate the interaction between human myoblast cells and the material. Employing a bioreactor in the stretching protocol for a more repeatable and sterile process is another opportunity for future research. Harnessing the developed stretching and seeding protocols in conjunction with the outlined future work will drive BD's future biomaterial and hernia mesh research forward, hopefully resulting in a clinically approved electrospun P4HB hernia mesh.

End Matter

Author Contributions and Notes

A.P. and J.M. designed research, A.P and J.M. performed research, A.P. and J.M. analyzed data; and A.P. and J.M. wrote the paper.

AP is a current intern of Becton, Dickinson, and Co.

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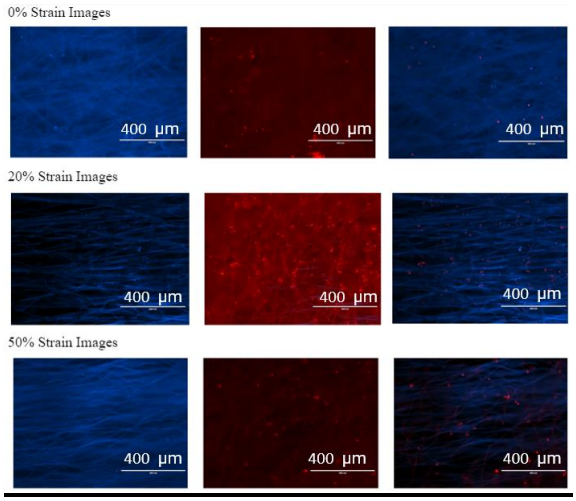
Appendix



Appendix Figure 1. Separate Color Channel Images for 0% Strain, 20% Strain, and 50% Strain. The blue channel images fluoresced by Dapi are on the left; the red channel images fluoresced by Propidium Iodide are in the middle; the overlay images are on the right, including both the red and blue channel.