IDENTIFYING AND REDUCING VARIABILITY, IMPROVING SCAFFOLD
MORPHOLOGY, AND INVESTIGATING ALTERNATIVE MATERIALS FOR THE
BLOOD VESSEL MIMIC LAB ELECTROSPINNING PROCESS

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by

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TITLE: Identifying and Reducing Variability, Improving Scaffold Morphology, and Investigating Alternative Materials for the Blood Vessel Mimic Lab Electrospinning Process

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ABSTRACT

Identifying and Reducing Variability, Improving Scaffold Morphology, and Investigating Alternative Materials for the Blood Vessel Mimic Lab Electrospinning Process

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The work of the Cal Poly Tissue Engineering Lab is primarily focused on the fabrication, characterization, and improvement of “Blood Vessel Mimics” (BVMs), tissue engineered constructs used to evaluate cellular response to vascular medical devices. Currently, cells are grown onto fibrous, porous tubes made using an in-house electrospinning process from PLGA, a biocompatible co-polymer. The adhesion and proliferation of cells in a BVM is reliant on the micro-scale structure of the PLGA scaffold, and as such it is of great importance for the electrospinning process to consistently produce scaffolds of similar morphologies. Additionally, it has been shown that cell proliferation increases with scaffolds of smaller fibers and pores than the current electrospinning protocol can produce. Finally, the Tissue Engineering Lab has interest in testing devices in more tortuous BVM bioreactor designs, however the use of relatively rigid PLGA scaffolds has severely limited the ability to construct more complicated vessel geometries.

The overall goal of this thesis was to improve fabrication and characterization of electrospun polymer scaffolds for BVM use. The specific aims of this thesis were to: 1) Improve scaffold characterization by comparing two techniques for fiber diameter measurement and implementing a technique for pore area measurement. 2) Reduce scaffold fiber diameter and pore area by investigating humidity and solvent composition electrospinning parameters. 3) Reduce process variability by developing a more specific electrospinning protocol. 4) Improve scaffold consistency and use by understanding and reducing PLGA scaffold shrinkage. 5) Identify and evaluate more flexible polymers as potential alternatives for electrospun BVM scaffolds.

In order to accomplish these aims, first, several BVM and outside literature images were taken and evaluated with current and prospective fiber diameter techniques, and with 2 prospective pore area techniques to characterize accuracy and consistency of each method. It was found that the prospective fiber diameter measurement technique was not superior to the current method. The techniques developed for pore area measurement were found to produce results that differed significantly from each other and from the published value for a given image. Next, changes to environmental and solution composition parameters were made with the hopes of reducing fiber diameter and pore area of electrospun PLGA scaffolds. Changes in relative humidity did not appear to significantly affect scaffold fiber diameter while changes to solvent composition, specifically the use of acetone, resulted in fibers significantly smaller than those regularly achieved in the BVM lab. Next, several sources of variability in the electrospinning

protocol were identified and subsequently altered to improve consistency and usability. Specifically, this included redefining the precision with which PLGA mass was measured, repositioning electrical equipment to reduce the effect of stray electrostatic forces on the polymer solution jet, attempting to control the temperature and humidity inside the electrospinning enclosure, and improving the ease with which scaffolds are removed from their mandrels through alternative mandrel surface treatments. In addition to overall process variability, the issue of scaffold shrinkage during BVM use was investigated and two possible treatments, exposure to either ethanol or elevated temperatures, were proposed based on previous electrospinning literature results. Each was tested for their effectiveness in mitigating shrinkage through exposure to BVM setup-mimicking conditions. It was found that both treatments reduced scaffold shrinkage compared to control samples when exposed to BVM setup-mimicking conditions. Finally, 3 flexible polymers were selected and electrospun to compare against typical PLGA results and to conduct a kink radius test as a metric for measuring flexibility as it pertains to the proposed BVM lab application. It was concluded that two types of thermoplastic polyurethane (tPU) were not acceptable electrospinning materials for use in the BVM lab. Additionally, while polycaprolactone (PCL) could be successfully electrospun it could not undergo the amount bending required for more tortuous BVM bioreactor designs without kinking.

Overall, the work in this thesis provided insight into multiple scaffold characterization techniques, reduced overall electrospinning variability in the fabrication and use of PLGA scaffolds, and defined processing parameters that have been shown to yield scaffolds with smaller morphological features than all prior Tissue Engineering Lab work. By creating better, more effective scaffolds, researchers in the Tissue Engineering Lab can more accurately mimic the structure and properties of native blood vessels; this, in turn, will result in BVM cell responses that more closely resemble that of native tissue. Creating consistent and appropriate BVMs will then lead to impactful contributions to the existing body of tissue engineering research and to better preclinical device testing.

Keywords: electrospinning, scaffold, PLGA, polymer, fiber diameter, pore area, variability, shrinkage, flexible, blood vessel mimic, tissue engineering

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TABLE OF CONTENTS

	Page
LIST OF TABLES	xvi
LIST OF FIGURES	xviii
1. INTRODUCTION	1
1.1 General Overview	1
1.2 BVM Overview	2
1.3 Tissue Engineering Overview	6
1.3.1 Desired Scaffold Characteristics	6
1.3.1.1 Mechanical Behavior	7
1.3.1.2 Degradation.....	7
1.3.1.3 Morphology.....	8
1.4 Polymer Science Overview	11
1.5 Electrospinning Overview.....	16
1.5.1 Electrospinning Parameters	20
1.5.1.1 Processing Parameters	20
1.5.1.2 Environmental Variables	22
1.5.1.3 Solution Variables.....	24
1.6 Previous BVM Lab Electrospinning Research.....	27
1.6.1 Colby James, 2009.....	27

1.6.2 Tiffany Peña, 2009	28
1.6.3 Yvette Castillo, 2012	28
1.6.4 Deven Patel, 2012.....	29
1.6.5 Toni Pipes, 2014.....	30
1.7 Summary and Aims of this Thesis	30
 2. COMPARISON OF SCAFFOLD MEASUREMENT METHODS AND IMPLEMENTATION OF AN ADDITIONAL CHARACTERIZATION TECHNIQUE	 34
2.1 Introduction	34
2.1.1 Fiber Diameter.....	34
2.1.2 Pore Area	37
2.2 Fiber Diameter Measurement Methods.....	41
2.2.1 Manual Method.....	43
2.2.2 DiameterJ Method	46
2.2.3 Comparisons	46
2.3 Pore Area Measurement Methods	48
2.3.1 Manual Image Thresholding.....	49
2.3.2 DiameterJ Segmentation and Mesh Hole Analysis	53
2.3.3 Comparisons	53
2.4 Fiber Diameter Results.....	55
2.4.1 Contrast Test.....	55

2.4.2 Reference Mesh Test	58
2.4.3 BVM Lab Images	59
2.4.3.1 Magnification Test.....	62
2.4.4 Literature Images	65
2.5 Pore Area Results	69
2.5.1 Contrast Test.....	71
2.5.2 Reference Mesh Test	72
2.5.2.1 Minimum Pore Area Restriction Test	73
2.5.2.2 Direct Measurement Method Comparison.....	76
2.5.3 BVM Lab Images	77
2.5.3.1 Magnification Test.....	81
2.5.4 Literature Images	83
2.6 Fiber Diameter Discussion	86
2.6.1 Contrast Test.....	86
2.6.2 Reference Mesh Test	87
2.6.3 BVM Lab Images	88
2.6.4 Literature Images	90
2.6.5 General Method Comparison.....	91
2.7 Pore Area Discussion	95
2.7.1 Contrast Test.....	95

2.7.2 Reference Mesh Test	97
2.7.3 BVM Lab Images	99
2.7.4 Literature Images	103
2.7.5 General Comparisons	104
2.8 Conclusion.....	105
3. FIBER DIAMETER AND PORE AREA REDUCTION STRATEGIES	109
3.1 Introduction	109
3.1.1 Previous BVM Lab Research	109
3.1.2 Current Research Focus	112
3.1.2.1 Environmental Parameters	112
3.1.2.2 Solution Parameters	114
3.2 Methods	116
3.2.1 Solution Mixing and Electrospinning	116
3.2.2 Environmental Parameters.....	116
3.2.3 Solvent Combinations.....	118
3.3 Results	119
3.3.1 Environmental Parameters.....	119
3.3.2 Solvent Combinations.....	126
3.4 Discussion	137
3.4.1 Humidity.....	137

3.4.2 Solvent	139
3.5 Conclusion.....	143
4. INVESTIGATING INCONSISTENCIES WITH THE CURRENT ELECTROSPINNING PROTOCOL.....	146
4.1 Introduction	146
4.1.1 Solution Mixing.....	147
4.1.2 Experimental Setup.....	148
4.1.3 Environmental Control	149
4.1.4 Scaffold Removal	150
4.2 Methods and Results	151
4.2.1 Solution Mixing.....	151
4.2.2 Experimental Setup.....	153
4.2.2.1 Eliminating Magnetic Interference	153
4.2.2.2 Environmental Effects	157
4.2.3 Scaffold Removal	160
4.2.3.1 Automotive Polish	161
4.2.3.2 Teflon Spray.....	162
4.2.3.3 Pre-Polished	162
4.2.3.4 4000 Grit Sanding	163
4.3 Discussion	163
4.3.1 Solution Mixing.....	164

4.3.2 Electrospinning Setup.....	164
4.3.3 Environmental Conditions.....	165
4.3.4 Scaffold Removal	166
4.4 Conclusion.....	170
5. INVESTIGATING AND MITIGATING SCAFFOLD SHRINKAGE	172
5.1 Introduction	172
5.2 Methods and Results	175
5.2.1 Differential Scanning Calorimetry	176
5.2.2 Stress Relief Treatments	179
5.2.3 Shrinkage Testing	182
5.2.4 Shrinkage Results	183
5.3 Discussion	189
5.3.1 Reasons for Shrinkage	189
5.3.2 Effect of Stress-Relief	190
5.3.3 Difference Between Chloroform	190
5.4 Conclusion.....	191
6. INVESTIGATION OF A FLEXIBLE ELECTROSPINNING MATERIAL	193
6.1 Introduction	193
6.1.1 Flexible Polymer Use in Vascular Tissue Engineering	196
6.2 Methods and Results	197

6.2.1 Materials Selection	197
6.2.1.1 Material Flexibility	198
6.2.1.2 Flexible Polymer Electrospinning Literature Review.....	200
6.2.1.3 Material Cost.....	205
6.2.1.4 Polycaprolactone.....	208
6.2.1.5 Thermoplastic Polyurethane	212
6.2.2 Electrospinning.....	214
6.2.2.1 Preliminary Trials	214
6.2.2.2 Second Flexible Polymer Trials.....	231
6.2.3 Characterizing Scaffold Flexibility	242
6.2.3.1 ePTFE	244
6.2.3.2 PLGA	245
6.2.3.3 PCL	247
6.3 Discussion	249
6.3.1 Materials Selection	250
6.3.2 Electrospinning.....	251
6.3.3 Kink Testing	251
6.4 Conclusion.....	256
7. DISCUSSION AND CONCLUSION.....	258
7.1 Summary and Aims of this Thesis	258

7.2 Challenges and Limitations	262
7.2.1 Pore Area Measurements.....	262
7.2.2 Environmental Conditions.....	262
7.2.3 Chloroform Expiration and Electrospinner Refurbishing	263
7.2.3.1 Old Electrospinning Collector	264
7.2.3.2 New Electrospinning Collector.....	265
7.3 Future Work	266
7.3.1 Reducing Fiber Diameter.....	266
7.3.2 Process Variability.....	267
7.3.3 Scaffold Shrinkage	268
7.3.4 Alternative Electrospinning Materials	268
7.4 Conclusion.....	270
REFERENCES	271
APPENDICES	
APPENDIX A: IMAGEJ PROTOCOL FOR FIBER DIAMETER MEASUREMENT	285
APPENDIX B: DIAMETERJ PROTOCOL FOR FIBER DIAMETER AND PORE AREA MEASUREMENTS	289
APPENDIX C: IMAGEJ PROTOCOL FOR PORE AREA MEASUREMENT	294
APPENDIX D: ORIGINAL BVM PLGA SOLUTION MIXING PROTOCOL.....	298
APPENDIX E: ORIGINAL BVM ELECTROSPINNING PROTOCOL	299
APPENDIX F: STANDARD OPERATING PROCEDURE FOR MIXING ACETONE AND CHLOROFORM FOR THE PURPOSES OF PLGA ELECTROSPINNING.....	301

APPENDIX G: REVISED BVM PLGA SOLUTION MIXING PROTOCOL	308
APPENDIX H: REVISED BVM ELECTROSPINNING PROTOCOL	310
APPENDIX I: SAMPLE CALCULATIONS WITH THE REVISED SOLUTION MIXING PROTOCOL (APPENDIX G)	314
APPENDIX J: ISO 7198:1998 KINK RADIUS TEST PROTOCOL	316

LIST OF TABLES

	Page
Table 1. Pixel-to-micron conversion ratio for SEM images	45
Table 2. Pore Area Measurement Image Descriptions	49
Table 3. Fiber Diameter Contrast Test Results	57
Table 4. Reference Mesh Fiber Diameter Results	58
Table 5. BVM Image Fiber Diameter Results	60
Table 6. Fiber Diameter Results for Magnification Test	64
Table 7. Fiber Diameter Results for Literature SEM Images	66
Table 8. Pore Area Contrast Test Results	71
Table 9. Pore Area Values for Reference Mesh Minimum Pore Area Restriction Test ...	75
Table 10. Pore Area Values for Reference Mesh Method Comparison	76
Table 11. BVM Image Pore Area Results	79
Table 12. Pore Area Results for Magnification Test	82
Table 13. Pore Area Results for Literature SEM Images	84
Table 14. Samples and Parameters for Environmental Parameters Trial	117
Table 15. Solvent ratios to achieve 15wt% PLGA electrospinning solution.....	118
Table 16. Samples and Parameters for Solvent Combinations Trial	119
Table 17. Fiber Diameter and Pore Area of Environmental Parameters Study, Spin 1E.....	122
Table 18. Fiber Diameter and Pore Area of Environmental Parameters Study, Spin 2E.....	125
Table 19. Fiber Diameter and Pore Area of the Solvent Combinations Study, Spin 1S	129
Table 20. Fiber Diameter and Pore Area of the Solvent Combinations Study, Spin 2S	131
Table 21. Fiber Diameter and Pore Area of the Solvent Combinations Study, Spin 3S	133
Table 22. Fiber Diameter and Pore Area of the Solvent Combinations Study, Spin 4S	135
Table 23. Mandrel Surface Finish Treatment Scores.....	161
Table 24. Descriptions for Scaffold Sections Spun for Stress-Relief.....	181
Table 25. Average Decrease in Scaffold Length and Diameter After Sterilization and Conditioning	183

Table 26. Materials Selection Criteria for Flexible Polymer Electrospinning.....	198
Table 27. Costs of Flexible Polymers for Electrospinning	206
Table 28. Costs of Proposed Solvents for Flexible Polymer Electrospinning	206
Table 29. As-Planned Preliminary Trial Parameters for Flexible Polymer Electrospinning	215
Table 30. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 3P	220
Table 31. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 4P	222
Table 32. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 5P	224
Table 33. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 6P	226
Table 34. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 8P	228
Table 35. Summary of Preliminary Flexible Material Study Fiber Diameter and Pore Area Results.....	229
Table 36. Follow-up Trial Parameters for Flexible Polymer Electrospinning.....	231
Table 37. Fiber Diameter and Pore Area of Second Flexible Material Study, Spin 1F..	233
Table 38. Fiber Diameter and Pore Area of Second Flexible Material Study, Spin 2F..	235
Table 39. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 3F	238
Table 40. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 4F	239
Table 41. Summary of Secondary Flexible Material Study Fiber Diameter and Pore Area Results.....	240
Table 42. Summary of Successful Flexible Material Study Fiber Diameter and Pore Area Results.....	240
Table 43. Summary of All Suggested Future Work	269

LIST OF FIGURES

	Page
Figure 1. Typical BVM bioreactor design. The electrospun scaffold is suspended in the middle of the chamber (A) and connected to luminal inlet (B) and outlet (C) ports, and adjacent to the extraluminal outlet port (D). Media flows from the reservoir (E) into a peristaltic pump (F) and through the scaffold, either lumenally (through B and C) or transmurally (through B and D) ⁷ .	4
Figure 2. An electrospun PLGA scaffold (top) sutured onto sterilizable fittings that interface with the luminal ports on the BVM chamber. The lower image shows a BVM chamber with scaffold in place before attachment to the media reservoir and peristaltic pump ⁸ .	5
Figure 3. Various types of native human ECM, including fibrosarcoma cancer cells (red) on a collagen (blue) matrix (top left), elastin ECM of an aorta (top right), several types of porcine small intestinal submucosa ECM (bottom left), and a fibrin ECM mesh with attached human leukocyte (bottom right) ²⁶⁻²⁹ .	9
Figure 4. SEM image of ePTFE at 1000x magnification, showing solid nodes connected by fibrous material ³⁰ .	10
Figure 5. Simplified model of molecules in an amorphous arrangement. Green atoms represent those which can only exhibit oscillatory motion, while the blue atom has an opportunity to move to a new location relative to other atoms due to a higher free volume ⁵⁹ .	13
Figure 6. Simplified PLGA copolymerization reaction featuring cyclic dimers of LA and GA and respective PLGA monomers. Sn(Oct) ₂ is Tin(II) 2-ethylhexanoate, a polymerization catalyst ⁷⁸ .	15
Figure 7. Diagram of a general model of an electrospinning setup. The collection target rotates and translates to ensure even, random coverage of polymer fibers ⁸¹ .	17
Figure 8. Formation and journey of a polymer jet beginning at the needle tip and depositing on a grounded surface ⁸⁵ .	18

Figure 9. Transition from ohmic to convective flow in an elongating polymer jet. Negative charges initially distributed throughout the solution travel to the surface of the jet ⁸⁷	19
Figure 10. Visualization of bending instabilities experienced during electrospinning (left) and a picture of a polymer experience bending instability during electrospinning (right) ^{88,89}	19
Figure 11. Detailed flow chart of the DiameterJ measuring technique and output ¹¹⁵	36
Figure 12. SEM image of a PLGA scaffold (left), and the same image after thresholding (right)	39
Figure 13. Woven 316L stainless steel mesh used as a reference sample for fiber diameter and pore area measurements	42
Figure 14. SEM images with increasing amounts post-processing contrast enhancement. Top is as-imaged, all other images represent increasing amounts of pixel saturation.....	44
Figure 15. Sample PLGA scaffold SEM image after manual ImageJ measurement, showing a 3x3 circle template with 1 fiber diameter measurement per circle.	45
Figure 16. Original SEM image of PLGA scaffold (top left). Contrast-enhanced SEM image (top right). Insufficient thresholding limit that improperly highlights effective pore region (bottom left). Image thresholded to an appropriate amount (bottom right).....	50
Figure 17. A. Representative 15wt% PLGA scaffold SEM image at 600x magnification showing instances of improper pore detection (circled). B. SEM image after manual thresholding. C. Pore outlines produced from Analyze Particle ImageJ with no changes to min./max. particle size or circularity. D. Pore outlines at 0.1 – 1.0 circularity. E. Pore outlines identified with a minimum pore area of 200 pixels ² (~10 μm ²). F. Pore outlines at 0.1 – 1.0 circularity and with a minimum pore area of 200 pixels ²	52
Figure 18. Montage images showing all segmentation options for 0% contrast enhancement (top) and 80% contrast enhancement (bottom), with the	

image before segmentation located in the top left corner of each montage.....	56
Figure 19. Best segmentation option for 0% contrast enhancement (left) and 80% contrast enhancement (right); the images are identical, showing contrast does not affect segmentation.	57
Figure 20. Sample SEM images of the stainless steel reference mesh at 80x (top) and 200x (top) along with segmented versions for DiameterJ evaluation.	58
Figure 21. Boxplot of fiber diameter reference mesh results. *, $p < 0.05$ between groups. ***, $p < 0.001$ between groups.	59
Figure 22. PLGA scaffolds from the BVM lab spun for this thesis (1,2), spun for Toni Pipe's thesis (3) and for Deven Patel's thesis (4) ^{6,114}	60
Figure 23. Boxplot of experimental fiber diameter values for PLGA_1 (top) and PLGA_2 (bottom).....	61
Figure 24. Boxplot of experimental fiber diameter values for sample 1T. \$ signifies difference compared to known value of $1.71 \mu\text{m}$, $p < 0.05$	62
Figure 25. Boxplot of experimental fiber diameter values for sample 1D. \$ signifies difference compared to known value of $2.52 \mu\text{m}$, $p < 0.05$. *, $p < 0.05$ between groups.	62
Figure 26. SEM and segmented images of PLGA_1 at 600x (top), 800x (middle), and 1000x (bottom).....	63
Figure 27. Boxplot comparison of PLGA_1 between measurement methods. # signifies difference from Manual, 600x, $p < 0.05$. ***, $p < 0.001$ between groups.	64
Figure 28. Boxplot comparison of PLGA_2 between measurement methods. # signifies difference from DiameterJ, 600x, $p < 0.05$. *, $p < 0.05$ and ***, $p < 0.001$ between groups.....	65
Figure 29. SEM images for fiber diameter measurements from various literature sources of electrospun gelatin (top), PLGA/collagen copolymer (middle), and PLLA (top) ^{49,120,121}	66

Figure 30. Boxplots for experimental fiber diameter results for Lit_1F (top), Lit_2F (middle), and Lit_3F (bottom). \$ signifies difference from known value, p<0.05. **, p<0.01 between groups.....	68
Figure 31. Example of a large area of an SEM image outline being marked as a single pore by manual ImageJ thresholding (top, marking in red) and its corresponding original image (bottom).	70
Figure 32. Boxplot representing pore area results for the contrast test. #, p<0.05 difference between all other groups. *, p<0.05 difference between groups.	72
Figure 33. Thresholded images and outlines of pores for the minimum pore area restriction test at 80x magnification, with minimums of 0 μm^2 (top), 10 μm^2 (middle), and 50 μm^2 (bottom).....	74
Figure 34. Pore area measurements of the reference mesh material at increasing minimum pore area restrictions for 80x, 120x, and 200x magnifications.	75
Figure 35. Boxplot of reference mesh pore area values for manual and DiameterJ methods at various magnifications. \$, p<0.05 difference between known value of 1089 μm^2 . #, p<0.05 difference between all other groups besides DiameterJ, 80x. @, p<0.001 difference between all other groups.	77
Figure 36. BVM lab images with pore accompanying pore outlines of PLGA_1 (top) and PLGA_2 (bottom).	78
Figure 37. BVM lab images with pore accompanying pore outlines, of 1T (top) and 1D (bottom).	79
Figure 38. Boxplots of experimental pore area values for PLGA_1 (top), PLGA_2 (middle), and 1T (bottom). **, p<0.01 difference between groups. ***, p<0.001 difference between groups.....	80
Figure 39. Boxplot of experimental pore area values for sample 1D. \$, p<0.05 difference from published value of 59.83 μm^2	81
Figure 40. Boxplot of PLGA_2 pore area measurements at 600x, 800x, and 1000x. \$, p<0.05 difference between Manual 600x. #, p<0.001 difference between Manual 600x and 800x. @, p<0.05 difference between Manual 1000x.....	83

Figure 41. SEM images for pore area measurements from various literature sources of electrospun PCL/PEO (top), gelatin (bottom left), and PCL (bottom right) ^{52,119} .	84
Figure 42. Boxplots of experimental pore area results for Lit_1P (top), Lit_2P (middle), and Lit_3P (bottom). \$, p<0.05 difference from known value. *, p<0.05 between groups. **, p<0.01 between groups.	85
Figure 43. Original SEM image with two representative segmented PLGA scaffold of similar segmentation; determining the most accurate is based on operator discretion.	92
Figure 44. Original SEM images overlaid with two representative segmented images showing several areas of imperfect segmentation.	93
Figure 45. Manually thresholded (top) and outlined (bottom) SEM images at 0% (left) and 5% contrast enhancement). While the thresholded images appear relatively similar there are noticeable differences between the outline images generated by ImageJ.	96
Figure 46. Representative thresholded SEM image of a PLGA scaffold (top) with accompanying pore outline from the ImageJ Analyze Particles tool (bottom). Specks on the outline image indicate small “pores” detected based on the thresholding process.	97
Figure 47. Example of pore area measurement presented in Deven’s thesis ¹¹⁴ .	100
Figure 48. Examples of other pore areas (yellow) in place of that shown in Figure 47 (white) that may be considered equally valid pore selections.	101
Figure 49. The BVM lab electrospinning setup with terrarium humidifier and controller in use.	117
Figure 50. SEM images of Spin 1E at 60x (left) and 600x (right) at proximal (top), middle (middle), and distal (bottom) positions along the scaffold.	121
Figure 51. Mandrel from Spin 1E after scaffold removal showing adhered patches of fibrous PLGA.	123
Figure 52. SEM images of Spin 2E at 60x (left) and 600x (right) at middle (top), and distal (middle) positions along the scaffold, as well as images at 50x	

(bottom left) and 60x (bottom right) that show disturbances where patches of scaffold tore out and remained on the mandrel.	124
Figure 53. Box and whisker plot of humidity trial fiber diameter results. Average fiber diameter did not differ significantly between 1E and 2E.....	125
Figure 54. Box and whisker plot of humidity trial pore area results. *, $p < 0.05$ between groups.	126
Figure 55. SEM images of Spin 1S at 100x (top left), 600x (top right), 1000x (bottom left) and 2000x (bottom right) all from the proximal position of the scaffold.....	129
Figure 56. SEM images of Spin 2S at 100x (left) and 600x (right) at the proximal position along the scaffold.....	131
Figure 57. SEM images of Spin 3S at 60x (left) and 600x (right) at proximal (top) and distal (bottom) positions along the scaffold.....	133
Figure 58. SEM images of Spin 4S at 60x (left) and 600x (right) at proximal (top) and distal (bottom) positions along the scaffold.....	135
Figure 59. Box and whisker diagram of solvent trial fiber diameter. \$, $p < 0.001$ between 3S. #, $p < 0.05$ between 4S. @, $p < 0.001$ between 4S.....	136
Figure 60. Box and whisker diagram of solvent trials pore area. \$, $p < 0.001$ between 4S. #, $p < 0.05$ between 2S. @, $p < 0.01$ between 2S.....	137
Figure 61. A (a) simulated and (b) real-world experimental example of the Plateau-Rayleigh instability occurring over time in a polymer suspended between 2 parallel plates ¹³³	141
Figure 62. Electrospinner orientation terms relative to the position of the electrospinning collector (left) and the position of the red charge-carrying wire during electrospinning (left).....	149
Figure 63. Image of the current electrospinning setup.	149
Figure 64. Comparison between the old (left) and new (right) positions of the red charged wire.....	154
Figure 65. SEM images and boxplots showing difference between proximal and distal scaffold wall thicknesses spun with the old wire placement. ***, $p < 0.001$ between groups.....	155

Figure 66. SEM images and boxplot of scaffold wall thickness spun with the new wire position There is no significant difference between scaffold locations at the new position.....	156
Figure 67. Image of the humidifier (top left), controller (top right), and entire setup inside the electrospinning fume hood and enclosure.	159
Figure 68. Example of a mandrel loaded into the electrospinning collector with the spring loaded-barrel on the distal end under no load (top) and under compression (bottom)	168
Figure 69. Sterilization of scaffolds using an ethanol submersion (top) and BVM setup images (bottom) showing the path of media flow through the scaffold and bioreactor via peristaltic pumping while in incubation conditions.....	173
Figure 70. Full DSC output for as-obtained 75:25 PLGA pellets (top) and the glass transition region (bottom) of the curve.	177
Figure 71. Full DSC output for electrospun 75:25 PLGA (top) and the glass transition region (bottom) of the curve.	178
Figure 72. Scaffold divided for stress relieving experiments (top), scaffold sections prepared for heat treatment (bottom left) and for ethanol treatment (bottom right).....	181
Figure 73. Scaffold sterilization treatment (left) and a heat treatment step inside a low-temperature furnace at 37°C (right) to mimic the BVM conditioning environment.	182
Figure 74. SEM images of scaffolds prior to heat treatment (top left), after heat treatment (top right), and after sterilization (bottom left) and conditioning-mimicking step (bottom right). The white artifacts are of unknown origin.	184
Figure 75. Scaffold section prepared for sterilization and conditioning (top) and a comparison of treated and untreated scaffold sections after sterilization and conditioning (bottom).	185

Figure 76. Plot showing average difference in scaffold length and diameter between stress-relieved and control samples after BVM sterilization and conditioning treatments.	186
Figure 77. DSC curve (top) and glass transition area (bottom) of electrospun PLGA scaffolds after heat treatment.....	187
Figure 78. DSC curve (top) and glass transition area (bottom) of electrospun PLGA scaffolds after ethanol treatment.....	188
Figure 79. Complex scaffold configurations designed by previous BVM lab researcher Dalton Chavez using ePTFE ⁸ . Electrospun PLGA would not be able to be formed into any of these geometries due to its lack of elasticity.	195
Figure 80. Plot of Young's modulus of several polymers used in biomedical application ⁵⁷	198
Figure 81. PCL pellet agglomerate after exposure to acetone.	217
Figure 82. Spin 3P scaffold (top scaffold) with partially melted then solidified distal end (left). Spin 4 is below as a reference.....	218
Figure 83. SEM images of Spin 3P at 60x (left) and 600x (right) at proximal (top), middle (middle), and distal (bottom) positions along the scaffold.....	219
Figure 84. Spin 4P with parallel lines superimposed to show the scaffold tapering in thickness.	221
Figure 85. SEM images of Spin 4P at 60x (left) and 600x (right) at proximal (top) and distal (bottom) positions along the scaffold.....	222
Figure 86. Spin 5P scaffold during removal from the mandrel. Peeling instead of smooth sliding made removal of a fully intact tubular scaffold impossible.....	224
Figure 87. SEM images of Spin 5P at 60x (left) and 600x (right) at proximal (top), middle (middle), and distal (bottom) positions along the scaffold.....	224
Figure 88. SEM images of Spin 6P at 60x (left) and 600x (right) at the proximal position along the scaffold.....	226
Figure 89. SEM images of Spin 8P at 60x (left) and 600x (right) at the proximal position along the scaffold.....	228

Figure 90. Box and whisker plot of preliminary flexible polymer scaffold fiber diameter results. *, $p<0.05$ between groups.	229
Figure 91. Box and whisker plot of preliminary flexible polymer scaffold pore area results. *, $p<0.05$; **, $p<0.01$; and ***, $p<0.001$ between groups.	230
Figure 92. SEM images of Spin 1F at 60x (left) and 600x (right) at proximal (top) and distal (bottom) positions along the scaffold. The features visible on the polymer areas in the 600x images are indicative of the surface finish on the mandrel used for electrospinning.	233
Figure 93. SEM images of Spin 2F at 60x (left) and 600x (right) at proximal (top) and distal (bottom) positions along the scaffold.	235
Figure 94. Spin 3F scaffold removal attempts, showing tearing and rolling (top), bunching and rolling (middle), and scraps cut from the mandrel after all methods had been exhausted.	237
Figure 95. SEM images of Spin 3F at 60x (left) and 600x (right) at the proximal position along the scaffold.	238
Figure 96. SEM images of Spin 4F at 60x (left) and 600x (right) at the proximal position along the scaffold.	239
Figure 97. Combined fiber diameter results from the preliminary and secondary flexible polymer trials. *, $p<0.05$ between groups. #, $p<0.001$ between all other groups.	241
Figure 98. Pore area results for the secondary flexible polymer trials. *, $p<0.05$ between groups. #, $p<0.001$ between all other groups.	241
Figure 99. ISO 7198:1998 kink radius templates.	244
Figure 100. Representative images of ePTFE tubing kink testing. ePTFE did not kink at any radius, including with the smallest circle templated of 7.5 mm radius.	245
Figure 101. Representative images of PLGA scaffold kink testing. Images on the left show the scaffold during testing, while images on the right indicate the permanent deformation observed after the respective template.	246

Figure 102. PLGA tink testing imgs with the 25 mm diameter template (top) with which kinking first occurred, and with the 13 mm diameter template (bottom).	247
Figure 103. Representative images of PCL scaffold kink testing. Kinking occurred immediately with all templates.	248
Figure 104. Manual flexion of an electrospun PCL scaffold. The scaffold was flexible and could be bent easily by hand (left), but experienced somewhat of a hysteresis effect when returning to its as-fabricated shape (right).	248
Figure 105. Tri-axial deformation and strain experienced by a tube in bending ¹⁸⁵	253
Figure 106. SEM images of the inside surface of ePTFE tubing from the BVM lab.	255
Figure 107. SEM images of ePTFE tubing experiencing contraction. The distance between nodes has decreased in areas of compression.	256

1. INTRODUCTION

1.1 General Overview

The focus of this thesis was to improve upon the processes for fabricating and characterizing electrospun scaffolds for use in the Cal Poly Tissue Engineering Lab as substrates for blood vessel mimic (BVM) constructs. BVMs are used as a form of pre-clinical intravascular medical device testing, and are central to the research done in the Tissue Engineering Lab. Several previous theses have been published on the topic of electrospinning, and this work is intended to build upon those to further improve the electrospinning process in the BVM lab. This work includes standardizing, improving, and expanding upon the current characterization techniques for electrospun scaffolds, improving scaffold characteristics by reducing average fiber diameter and pore size, reducing or eliminating sources of variability in the electrospinning process, investigating and reducing scaffold shrinkage *in vitro*, and exploring options for flexible polymer systems to replace PLGA for use in more complex BVM designs, each of which will be covered in-depth in the following chapters.

The following introduction sections provide relevant background information and research concerning the history and relevancy of the BVM system, the role of the scaffold in tissue engineering and in BVMs specifically, and the process of electrospinning and how various processing parameters and solvent properties can impact scaffold properties and morphology. This is presented alongside a summary of previous

Tissue Engineering Lab electrospinning theses to provide a basis for understanding and interpreting the rationale, methods, and results provided in this thesis.

1.2 BVM Overview

The Tissue Engineering Lab at Cal Poly focuses its research into the field of tissue engineering, specifically to create structures that resemble human blood vessels, or BVMs. In general, tissue engineering can be defined as the deliberate combination of cells, a scaffold on which to affix and grow cells, and a biologically stimulating environment to create functional tissues for the purposes of repairing, sustaining, or augmenting existing bodily tissues¹. In this way, researchers and tissue engineers hope to access the natural tissue-generating and maintaining ability of cells and biological systems by providing them with a favorable environment in which to flourish².

Tissue engineering is commonly conducted to alleviate the problems facing more traditional treatment options such as receiving donor tissue (allografts), autografts, and medical devices. The most evident and recurring issue with using donor tissue to treat currently-ailing patients is a chronic shortage of donors and an ever-increasing waitlist of patients in need; As of July 2017, 110,000 patients are listed on the national transplant waiting list, while only 33,611 transplants were performed in the previous year³. There also exists the ever-present problem of tissue rejection and navigating the patient's immune response to foreign bodies. Autologous tissue transplants also present several limitations: If a patient is suffering from a genetic ailment then the transplanted tissue will have similar defects, limiting the effectiveness of the procedure. Secondly, the act of

removing tissue to be grafted elsewhere takes a toll on the patient, especially if they are already ailing from their current condition. In the case of coronary bypass, for example, sections of blood vessel are removed from healthier portions of the body such as the arms, legs, or chest, causing some amount of injury in those locations and withdrawing from the finite supply of potential donor tissue in the patient that may be needed in case of a subsequent procedure^{4,5}. By fabricating tissue from cells that have been grown and expanded in a laboratory setting, the physical burden on the patient may be lessened.

The BVM lab at Cal Poly focuses on the 3 main aspects of tissue engineering in some capacity, by experimenting with various types of cells, scaffold fabrication techniques, and biologically stimulating environments to produce the most viable blood vessel-mimicking structures.

While many institutions take part in tissue engineering research and development for the purposes of eventually developing a construct that can be implanted into a patient, the Cal Poly BVM lab is focused on continually improving an *in vitro* blood vessel construct. This is done for the purposes of measuring cellular responses when exposed to medical devices for the purposes of pre-clinical device testing. These pre-clinical trials are performed prior to animal-based testing to reduce the high costs and variability sometimes associated with animal test results⁶.

The BVM model consists of human umbilical vein endothelial cells (HUVEC), human umbilical artery smooth muscle cells (HUASMC), or a combination of HUVECs

and HUASMCs cultured onto a polymeric scaffold. This scaffold is most commonly fabricated from a poly(lactic-*co*-glycolic acid) copolymer via an in-house electrospinning process. Electrospinning creates a randomly arranged fibrous, porous structure onto which the cells can adhere. The cell proliferation and growth occurs in a bioreactor designed within the BVM lab (Figure 1 and 2).

Figure 1. Typical BVM bioreactor design. The electrospun scaffold is suspended in the middle of the chamber (A) and connected to luminal inlet (B) and outlet (C) ports, and adjacent to the extraluminal outlet port (D). Media flows from the reservoir (E) into a peristaltic pump (F) and through the scaffold, either luminally (through B and C) or transmurally (through B and D)⁷.

Figure 2. An electrospun PLGA scaffold (top) sutured onto sterilizable fittings that interface with the luminal ports on the BVM chamber. The lower image shows a BVM chamber with scaffold in place before attachment to the media reservoir and peristaltic pump⁸.

Currently the Tissue Engineering Lab utilizes rigid, straight-walled tubular scaffolds for BVM setups, however flexible scaffold material such as ePTFE has been used for more complex vessel paths in the past^{8,9}. Chapter 6 of this thesis will discuss this matter in greater detail. The next sections of this chapter will overview the role of the scaffold in tissue engineering, as well as the materials commonly used to form scaffolds and their desired properties.

1.3 Tissue Engineering Overview

As stated earlier, a large portion of tissue engineering involves the use of a scaffold on which researchers grow cells. This combination of cells and scaffold, a *construct*, is exposed to an environment that facilitates cell growth and proliferation. This environment can be provided in a laboratory setting or *in vivo* to take advantage of the natural facilitation of biologic processes¹⁰. This scaffold is typically fabricated and/or processed into a shape like that of the tissue being grown (the shape of a tube for a blood vessel, for instance), and is designed such that the characteristics of the scaffold most closely mimic those of native tissue; Ideally scaffolds fabricated using engineering materials would act identically as native extracellular matrices in terms of chemical and mechanical properties, however this is rarely the case. Identifying and implementing these desired attributes in an engineered scaffold is one of the key hurdles in creating consistently successful tissue constructs.

1.3.1 Desired Scaffold Characteristics

Any scaffold that is used for a tissue engineering application is made of one or more biomaterials, broadly defined as any single or combination of synthetic and natural materials that are used to treat, augment, or replace tissues and functions in the body^{1,10,11}. Specifically, these materials must fulfill several stringent requirements with regards to mechanical behavior, degradation, physical morphology, and others such as biocompatibility and processability to successfully integrate with the body and to be a realistic option for a tissue engineering scaffold.

1.3.1.1 Mechanical Behavior

Biomaterials used for tissue engineering scaffolds must have mechanical strengths and stiffness close or equal to that of the native tissue they are replacing once they are in scaffold form. Because scaffolds must nearly always exist as porous structures, their mechanical behavior can be more difficult to predict. Materials that are too weak or too compliant may fail before the body can bolster or replace it with native tissue, however some compliance is required especially when mimicking soft tissues like blood vessels¹². The vast majority of tissue engineering scaffolds are made from polymers, and as such the mechanical properties can be altered by co-polymerizing different constituent materials to yield a blend that utilizes properties of its components, like the many different types of PLGA, a biomaterial co-polymer of poly(lactic acid) (PLA) and poly(glycolic acid) (PGA)¹³. Flexible polymers such as polycaprolactone, polyurethane, and collagen are often used to form such scaffolds, and are employed in applications like tissue engineered blood vessels, neural structures, and skin^{14–21}.

1.3.1.2 Degradation

Devices and materials that are implanted within the body face harsh, unforgiving conditions that can cause significant degradation over time; extreme pH, fatigue, electrolytic bodily fluids, and bodily immune response can all lead to degradation^{22,23}. This can be detrimental to devices that are intended to live with the patient for the rest of their life, however tissue engineering applications take advantage of this phenomenon by designing materials to degrade over a time period similar to the time required for the body to replace it with native tissue²⁴. For instance, poly(lactic acid) (PLA) and

poly(glycolic acid) (PGA) are often combined to form poly(lactic-*co*-glycolic acid) (PLGA) of various molecular ratios. While PLA resists water uptake and hydrolysis due to its hydrophobicity and the crystalline nature of PGA limits the access of water to most the polymer backbone, PLGA exhibits a more hydrophilic nature than PLA, and experiences a sharp drop in maximum crystallinity compared to PGA with increasing PLA content. In this way, varying the relative amounts of constituent material in PLGA will result in a wide range of degradation times when used in bodily conditions²⁴. Biodegradable polymers can be sourced directly from or be derived from natural sources and include polymers such as collagen, elastin, polyhydroxyalkanoates, and cellulose, or can be formed synthetically, including poly(ϵ -caprolactone) (PCL), PLA, PGA, and many others²⁴.

1.3.1.3 Morphology

In addition to matching the mechanical performance of a native tissue, scaffolds must also replicate an environment favorable to cell adhesion and proliferation. This is primarily done by processing the material in such a way that features on the micro- or nano-scale form sites at which cells can adhere, commonly through the formation of pores. Pores are created in attempts to simulate the naturally-occurring extracellular matrix (ECM) that surrounds and houses cells (Figure 3)²⁵.

Figure 3. Various types of native human ECM, including fibrosarcoma cancer cells (red) on a collagen (blue) matrix (top left), elastin ECM of an aorta (top right), several types of porcine small intestinal submucosa ECM (bottom left), and a fibrin ECM mesh with attached human leukocyte (bottom right)^{26–29}.

The synthetic material expanded polytetrafluoroethylene (ePTFE) can be used as a non-degradable biomaterial for vascular tissue engineering due to its microscopic morphology of nodes connected by strands of fiber that provide pores for cells to inhabit (Figure 4).

Figure 4. SEM image of ePTFE at 1000x magnification, showing solid nodes connected by fibrous material³⁰.

In addition to obtaining pre-manufactured ePTFE for research, it is possible to fabricate porous scaffolds with in-house techniques, such as electrospinning. A significant amount of research has been done on tailoring the morphology of electrospun polymeric scaffolds, specifically the size and shape of pores and fibers, to best accommodate cells during seeding and culture^{31–40}. A holistic understanding of the interplay between polymer and solvent properties and processing parameters is necessary to properly tailor the resulting properties of a scaffold fabricated in-house; a background on polymer science and its pertinence to electrospinning specifically will be covered in detail in the following sections.

1.4 Polymer Science Overview

Polymers as a materials class have significant physical and chemical diversity and can be precisely tailored for countless applications⁴¹. To match the mechanical properties of the many flexible tissues in the body when designing implantable biomedical solutions, polymers are frequently considered for long-term implantable applications^{42–45}. Additionally, polymers are utilized for their ability to be reliably broken down in the presence of a biological environment, allowing for their use in temporary, degradable implants in which the polymeric structure is naturally replaced by biologic material^{20,41,46–48}. In tissue engineering applications, polymers are typically used as a scaffold on which cells are grown and proliferated. This synthetic substrate acts as a replacement for the natural extracellular matrix (ECM), a complex network of natural materials that provide physical and chemical stimulus for cells throughout the body^{25,33,49–53}.

While the requirement of being formed in a porous or otherwise ECM-mimicking geometry is one primary requirement for most polymeric biomaterials, several others exist as well: Polymers must endure a sterilization process, the constant contact with a corrosive, aqueous environment, the elevated temperature of the body, and must retain its mechanical properties throughout the duration of its useful life. Typical sterilization processes include autoclave, electron beam, ethylene oxide (EtO) exposure, and gamma radiation, all of which can cause some polymers to melt, deteriorate, or embrittle to the point of uselessness^{54–56}. Additionally, the processing of a material into a porous structure can cause changes in mechanical and chemical properties that must be considered when

choosing a material for a tissue engineering application. For example, according to CES Bioengineering EduPack materials selection software, the Young's modulus for PLGA exists as a range between 1.25 and 2.85 GPa, while PTFE exhibits values between 0.4 and 0.552 GPa⁵⁷. However, in a previous Tissue Engineering lab thesis it was discovered that electrospun PLGA scaffolds and ePTFE tubing exhibited Young's modulus values of 13.251 MPa and 7.801 MPa, respectively, much lower than the published values in CES. This may be attributed to the fibrous, porous structure of the material, yielding most measurements of cross sectional area inaccurate without void content taken into account.

In addition to mechanical behavior, other properties inherent to the polymer structure such as glass transition temperature (T_g) may change or be expressed differently once processed or exposed to bodily conditions. Simply put, the glass transition of an amorphous or “glassy” polymer is the point at which the molecular chains have sufficient energy to move past each other, and the bulk material exhibits a “rubbery” behavior. Polymer chains and the atoms that make them up experience localized movement, oscillations due to their thermal energy, that create a certain amount of “free volume” between molecules (Figure 5)^{58,59}.

Oscillatory motion (vibrations):

Occurs within a “cage” formed by nearest neighbours. This is “solid-like” motion and is associated with small free volume.

Translational motion (diffusion):

Molecules jumps to new positions. This is “liquid-like” motion and is associated with large free volume.

Figure 5. Simplified model of molecules in an amorphous arrangement. Green atoms represent those which can only exhibit oscillatory motion, while the blue atom has an opportunity to move to a new location relative to other atoms due to a higher free volume⁵⁹.

The glass transition temperature represents the point at which the molecules contain enough thermal energy to oscillate in such a way that can move from their previous local focus of oscillation and occupy a new space, moving relative to other molecules to do so. The movement of an entire chain would not be energetically favorable even above the glass transition temperature, and so the movement of individual atoms occurs by rotations in small portions of the chain⁶⁰. The energy required to rotate a chain at a particular atomic bond is dictated by the presence of bulky molecules and side groups attached to the backbone; This explains why the glass transition temperature of polystyrene, which contains a large aromatic ring, is much higher than that of PLA, PGA, or PLGA (116 °C compared to 50-60, 35-40, and 50-55 °C, respectively)⁶⁰⁻⁶².

The existence of a glass transition is important for tissue engineering not only because it signifies a point at which amorphous polymers transition from relatively rigid and glassy to flexible and rubbery, but also because the effective glass transition

temperature can change due to processing. There have been multiple published instances of electrospinning resulting in a depressed T_g compared to a bulk sample of the sample material^{63,64}. Additionally, literature suggests that the processing of polymers into fibers and thin films depresses the glass transition temperature significantly. Polymer chains at a surface have greater latent free volume and thus a lower T_g and the formation of films and fibers drastically increases the surface area-to-volume ratio, such that the overall T_g of the structure is lowered as well with decreasing fiber diameter or film thickness^{65–67}. This can drastically change the mechanical properties of a material if its bulk T_g exists closely above the working temperature for an application that requires a thin film or micro-/nanofibrous structure. The depression of glass transition temperature of PLGA due to electrospinning has been shown to cause shrinkage in fibrous scaffolds prepared for various tissue engineering research efforts and has been experienced in the BVM lab^{63,64,68,69}.

For the past 8 years the polymer of choice for blood vessel scaffolds in the Cal Poly BVM lab has been poly(lactic-*co*-glycolic acid) (PLGA)^{6,70,71}. PLGA, along with its constituent materials, is frequently used in biodegradable biomedical implant applications. The BVM lab had previously obtained scaffolds of expanded polytetrafluorethylene (ePTFE), however due to their high cost and mismatched mechanical properties with native vessels an alternative material that could be fabricated and tailored in-house was considered^{7,70}. PLGA was selected due to its favorable biocompatibility and degradation, as well as mechanical properties similar to those of native vessels and evidence of adequate endothelial cell attachment⁷⁰. PLGA is used as a

biomaterial for several tissue engineering research applications including cartilage, bone, and blood vessels^{72,73}. It has also been approved by the FDA for use in several biomedical implants and drug products like suture reinforcement, skin grafts, and bone plugs^{74–76}.

PLGA is synthesized via ring-opening co-polymerization of the cyclic dimers lactide and glycolide⁷⁷. The Cal Poly BVM lab specifically uses a 75:25 ratio of lactide and glycolide that is a random copolymer with both L and D lactide isomer groups (Figure 6).

Figure 6. Simplified PLGA copolymerization reaction featuring cyclic dimers of LA and GA and respective PLGA monomers. Sn(Oct)_2 is Tin(II) 2-ethylhexanoate, a polymerization catalyst⁷⁸.

PGA is a highly crystalline polymer whereas poly(D,L lactic acid) (PDLLA, polymer constructed of both PLA isomers) is fully amorphous; when copolymerized the resulting PLGA exhibits a sharp drop in maximum crystallinity as PLA content increases, such that 75:25 PLGA is fully amorphous. The ability to tailor both crystallinity and hydrophobicity/philicity based on the relative amounts of PLA and PGA allows one to alter the degradation properties of PLGA to fit degradation timelines of less than 1

month, between 1 and 6 months, and beyond 6 months^{50,79}. In addition to degradation characteristics, the impact of relative polymer composition on solution parameters and on solvent compatibility all must be considered when selecting the most appropriate polymer for electrospinning; the following section discusses the effects of several electrospinning parameters including those dictated by polymer and solution properties on the electrospinning process.

1.5 Electrospinning Overview

Electrospinning is a polymer processing technique that uses electrostatic forces to draw out polymer fibers and deposit them on a conductive surface. The most common implementation of this idea is achieved by dissolving said polymer in an appropriate solvent, however some studies have shown success in electrospinning from a polymer melt⁸⁰. The polymer solution is then expelled from the syringe through a conductive needle charged via a high voltage power supply and pointed towards a grounded conductive collecting surface located some distance away from the needle tip (Figure 7).

Figure 7. Diagram of a general model of an electrospinning setup. The collection target rotates and translates to ensure even, random coverage of polymer fibers⁸¹.

Electrospinning is possible due to the combination of electrostatic forces and surface tension working on the polymer solution. As the solution is expelled from the syringe, a bead forms at the tip of the needle. This bead is held together by surface tension, however once the power supply is engaged the polymer serves as a conduit to complete the open circuit and the electrostatic forces deform the bead into a Taylor cone^{82–84}. Electrostatic forces overcome those of surface tension once critical voltage is reached, at which point a jet of solution erupts from the Taylor cone and travels towards the grounded collector (Figure 8).

Figure 8. Formation and journey of a polymer jet beginning at the needle tip and depositing on a grounded surface⁸⁵.

As the solution travels towards the collector it elongates and becomes thinner, beginning the formation of micro/nanofibers. The mechanism by which these fibers begin to form is the phenomenon of ohmic flow, in which the bulk of the polymer jet contains charges which are attracted to the grounded mandrel. However, as the jet thins and charges migrate to the surface of the jet after initial elongation, the charges begin to repel one another; this is a transition in current flow regime from ohmic flow to convective flow (Figure 9)⁸⁶.

Figure 9. Transition from ohmic to convective flow in an elongating polymer jet. Negative charges initially distributed throughout the solution travel to the surface of the jet⁸⁷.

The distribution of forces onto the surface of the jet induces what is referred to as bending instability, in which the repulsion of like charges causes the jet to whip and elongate to a much greater degree (Figure 10)⁸⁸.

Figure 10. Visualization of bending instabilities experienced during electrospinning (left) and a picture of a polymer experience bending instability during electrospinning (right)^{88,89}.

This phenomenon is dictated by repulsive Coulombic forces and causes fibers to bend and further elongate as the solvent evaporates; solvent evaporation is responsible for solidifying the fibers as they are deposited onto the collector^{90–92}.

To understand the process of electrospinning from a functional, application-based perspective, the following information describes the effect of several electrospinning parameters on the phenomena dictating electrospinning, and what impact they have on the resulting scaffold.

1.5.1 Electrospinning Parameters

There is a significant, complex relationship between the numerous electrospinning parameters and the outcome they have on the extent to which the polymer jet elongates, bends, and deposits to form a fibrous structure. What follows is a summary of many of the factors that contribute to the morphology and performance of an electrospun structure.

1.5.1.1 Processing Parameters

Processing parameters refer to those factors within an electrospinning setup that are controlled by the user and are independent of any specific solution attributes.

Gap Distance: Gap distance refers to the distance between the needle tip and grounded collector, and can be thought of as the travel distance of a polymer jet as it transitions from liquid bead to solid polymer fiber. There exists a critical distance range

for a given solution in which proper, consistent fiber formation is possible, and within this distance there is a general trend of decreasing fiber diameter with increasing gap distance^{93,94}. Electrospinning with gap distances smaller than this range will provide insufficient opportunity for solvent evaporation and fiber elongation, leading to undesirably large fibers or amorphous, non-fibrous structures⁹⁵. Gap distances greater than the critical range have been reported to produce beaded fibers, general considered to be unfavorable for most electrospinning applications⁹⁶.

Volumetric Flow Rate: The flow rate of a given electrospinning solution has similar characteristics to trends experienced with gap distance: there exists a range in which smooth fibers are produced, and flow rates above the upper limit of this range result in fibers with significant beading due to incomplete drying of the polymer via solvent evaporation⁹⁵. Flow rates below the lower critical value result in intermittent jet formation because significantly more solution is leaving the needle tip than is being replenished. Within the acceptable flow rate range there is a general trend of increasing fiber diameter with increased flow rate⁹⁶. The acceptable range of electrospinning flow rates is determined by the polymer/solvent combination and must be balanced with other parameters such as the applied voltage.

Applied Voltage: The application of a voltage to a conductive needle tip is essential to the electrospinning process, as it serves two key functions: It guides the polymer jet towards the grounded collector and it is required to overcome the forces of surface tension holding the solvent in a droplet within the needle. However, there is no

consensus on the effect changes in applied voltage on fiber and pore size⁹⁴. There are several instances of increased fiber diameter with increasing voltage due to an overall increase in polymer expelled for a given period^{83,97}. Others cite instances of decreasing fiber diameter with increased voltage, suggesting that there also exists an increase in repulsive Coulombic forces on the solution jet which result in more bending and whipping, stretching and narrowing fibers^{93,95}. Previous BVM lab work has shown that the used of PLGA in chloroform has shown a relatively weak correlation between increasing voltage and increasing fiber diameter⁷¹.

1.5.1.2 Environmental Variables

Environmental variables are those of the ambient environment in which the electrospinning is taking place, and include factors like temperature, humidity, and ambient pressure. These all impact the rate at which solvents evaporate and have a variety of effects on polymer processing in general⁹⁶.

Ambient Temperature: It is a well-known phenomenon that viscosity of most liquids is typically decreased with increasing temperature; this is due to the increased energy and resulting oscillation of molecules within the liquid, which reduces the force required to shear the sample⁹⁸. The stretching and elongating of fibers formed during the electrospinning process is caused by liquid shearing, and is resisted by the viscous forces of the solution. Therefore, increases in temperature typically result in fibers of smaller diameter due to the decreased viscous forces in the solution compared to said forces at lower temperatures⁹⁸. This has been corroborated by multiple electrospinning sources,

which have noted decreases in average fiber diameter with increases in temperature^{99–101}. However, it has also been noted that large increases in temperature can increase evaporation rates, solidifying fibers before they have elongated fully and artificially shortening the total fiber elongation time^{100,101}.

Relative Humidity: Similar to the effects of ambient temperature, the relative humidity of an electrospinning chamber impacts several aspects of the processing. Low relative humidity values have been found to increase solvent evaporation rates, truncating the time available for polymer jet elongation and resulting in fibers of larger diameter⁹⁶. However, high humidity has also been found to produce larger fibers due to the neutralization of charges on the polymer jet surface, decreasing the conductivity and ultimately the electrostatic force that is responsible for elongating the jet⁹⁶. It has also been observed that increased relative humidity results in increased water absorption within a polymer jet during electrospinning, increasing fiber diameter as well^{101,102}. One particular study only observed this effect in one of two polymers tested, cellulose acetate (CA); the other polymer, polyvinylpyrrolidone (PVP) exhibited smaller fibers with increased relative humidity, assumed to be caused by absorption of water, slowing solidification, and allowing for longer elongation times¹⁰¹. From these results, it is clear that humidity certainly has an effect on electrospinning results, however the specific impact on a particular solution is dependent on other parameters such as ambient temperature, solvent properties, and hygroscopic behavior of the polymer solution¹⁰³.

1.5.1.3 Solution Variables

Finally, there are attributes of the electrospinning process that are directly controlled through manipulation of the polymer solution used, such as solvent and polymer choice. These choices dictate solution properties like conductivity and dielectric constant that have shown correlation with various trends in scaffold and fiber morphology.

Conductivity: Solution conductivity is a measure of how readily the polymer solution will conduct electricity after application of the voltage source, and is determined by polymer and solvent properties as well as any other additives in the mixture. Many natural polymers used in electrospinning are polyelectrolytic, increasing the charge-carrying ability of the solution. Solution conductivity can be tailored for a specific application with the addition of ionic salts and surfactants, and solvents of varying conductivities^{96,104}. In general, increasing solution conductivity correlates to a decrease in fiber diameter; this is due to the increase in volume of like charges on the surface of a polymer jet and their repulsion to one another, elongating the jet more than an equivalent solution of lower conductivity^{105,106}.

Solution Concentration: Solution concentration refers to the relative composition of an electrospinning solution, typically reported in terms of wt.% for solid constituents and volume ratio for instances of multiple solvents. Several studies have shown that there is a minimum polymer concentration for a given polymer-solvent system that allows the formation of consistent, continuous fibers; below this threshold

there is a tendency to form beads or beaded fibers^{96,107}. Above this threshold there is a consistent trend of increasing fiber diameter with increasing polymer concentration in solution due to the increase in viscosity responsible for resisting the shearing of solution caused by elongation¹⁰². The concentration threshold represents a point at which the polymer chains are sufficiently entangled such that the solution cannot be pulled into a bead shape under the forces of surface tension^{96,108}.

Surface Tension: The surface tension of a polymer solution is largely dictated by the composition of the solvent used, and is responsible for the formation of beaded fibers¹⁰⁹. The forces of surface tension attempt to reduce the total surface area of the polymer jet during electrospinning and oppose viscoelastic forces in doing so; at low viscosities (typically achieved by lowering polymer concentration) beaded fibers form. For a given polymer concentration that produces beaded fibers, the composition of the solvent can be altered to form smooth fibers through the incorporation of other solvents with lower surface tensions^{83,96,109}. Additionally, surfactants can be added to existing solvent compositions to drastically lower surface tension values^{110,111}. Decreasing surface tension also provides the added effect of lowering the threshold voltage needed to form a jet from a bead of solution, resulting in a lower average fiber diameter and increasing the “electrospinnability” of solution compositions originally impossible to spin¹¹⁰.

Solution Viscosity: Solution viscosity is greatly influenced by the viscosity of the chosen solvent and the concentration of the polymer in solution. Viscosity refers to the resistance of a liquid to shear forces; this includes the electrostatic forces attempting to

stretch and elongate a polymer jet during electrospinning, and so typically a higher solution viscosity will prevent some degree of elongation and result in fibers with larger average diameter¹¹². Previous literature results show that below a minimum viscosity, typically controlled by changing the polymer concentration of a given polymer-solvent combination, beaded fibers are formed due to a lack of resistance to surface tension pulling the solution into droplets. Above this minimum viscosity smooth fibers are formed with increasing diameter as viscosity increases¹⁰². This is due to the aforementioned resistance to solution shearing inherent to the stretching and elongation of the polymer jet during electrospinning.

Polymer Molecular Weight: Polymer molecular weight is one component of solution viscosity, and is thus similarly important in dictating electrospun fiber results. Increases in molecular weight increase chain entanglement and provide more resistance to polymer chain alignment and fiber elongation¹⁰⁸. Similar to viscosity, molecular weight below a particular minimum threshold results in beads or beaded fibers. Above this threshold smooth fibers form, increasing in diameter and/or changing geometry into micro-ribbons⁹⁶. Molecular weight appears to share a relationship with concentration, since they both contribute to the overall density of -mer units within a solution, the viscous forces they generate. For instance, while most electrospinning is done between 5 and 15% concentration with polymers of >50,000 Mw values, McKee et al. were able to form fibrous electrospun structures from lecithin, a mixture of neutral lipids and phospholipids, in a 35wt% solution¹¹³.

The process of polymer electrospinning is the exclusive fabrication technique for scaffolds in the Cal Poly BVM lab, and is a common technique for creating porous, non-woven polymer structures with micro- or nano-scale features for a variety of research purposes. Electrospinning allows for a great deal of flexibility regarding compatible materials and possible scaffold geometries. For the purposes of the BVM lab, the aim of scaffold fabrication is to create tubular structures that mimic the size, shape, and morphology onto which various vascular cell types will adhere most effectively. The following section will outline relevant electrospinning work done in the Tissue Engineering to establish a foundation of information onto which the experiments of this thesis will be based.

1.6 Previous BVM Lab Electrospinning Research

Several prior Tissue Engineering Lab theses have focused on establishing the electrospinner, improving its capacity to fabricate scaffolds, and optimizing various parameters to yield the most effective scaffolds for BVM use. This section consists of a summary of these works as an introduction for the experiments described later in this thesis.

1.6.1 Colby James, 2009

Colby James was responsible for finding a suitable, in-house fabrication technique to replace pre-manufactured ePTFE tubing for the purposes of making BVM scaffolds. Electrospinning was chosen due to its ability to mimic native ECM by creating

a highly tailorable nanofiber mesh. Preliminary electrospinning trials were performed with a 90:10 copolymer of poly(lactide-*co*-caprolactone) [P(LLA-CL)] dissolved in chloroform; these results yielded a set of parameters that were used to in a consistency study to determine the variability in scaffold fiber diameter, wall thickness, and Young's modulus. The average fiber diameter obtained from the consistency study ranged from 6 to 9 μm and was significantly different between multiple scaffolds, as was wall thickness⁷.

1.6.2 Tiffany Peña, 2009

Tiffany Peña's work focused on selecting an appropriate material for long-term use with the BVM lab electrospinning technique, developing an optimized protocol for said material, and investigating its efficacy in a BVM setup, cultured with human umbilical vein endothelial cells (HUVECs). PLGA was ultimately selected due to prior evidence of adequate endothelial cell attachment, controlled degradation, biocompatibility, and mechanical properties similar to those of native vessels. Through several spins, Tiffany developed the set of electrospinning parameters used in the current BVM protocol, and observed un-beaded fibers that ranged in diameter between 5 and 6 μm . BVM setup results showed that HUVECs were able to penetrate the luminal surface and adhere to the scaffold, however a confluent cell lining was not observed⁷⁰.

1.6.3 Yvette Castillo, 2012

The work of Yvette Castillo was focused around establishing an understanding of the interactions between various electrospinning parameters and using these interactions to reduce the average fiber diameter, ideally to the range of 100-200 nm. Yvette spun

several scaffolds of varying solution concentration, gap distance, flow rate, and applied voltage, and achieved a fiber diameter of 2.74 μm , significantly lower than any previous BVM lab results. Several samples mixed with the lowest polymer concentration did not yield successfully spun scaffolds, and so the parameter was omitted from further analysis. The design of experiments and following regression analysis suggested that the strongest predictor of fiber diameter was flow rate, with which fiber diameter had a positive correlation. The model also suggested that gap distance and voltage had inverse relationships with fiber diameter, however it was acknowledged that a minimum voltage is necessary to overcome surface tension and form a jet⁷¹.

1.6.4 Deven Patel, 2012

The aims of Deven Patel's thesis were to upgrade the BVM lab electrospinning system, to develop a specific electrospinning protocol for the Blood Brain Barrier (BBB) model, and characterize the variability of scaffolds produced via this protocol. The electrospinner was outfitted with a new high voltage power supply allowing for negative polarity, a safer electrical layout, and a new syringe pump with an accompanying stand. Deven's BBB electrospinning protocol differed slightly from the one developed by Toni, using the optimal flow rate of 4.5 ml/hr as determined by Yvette's thesis along with an increase in voltage to 18kV. This protocol resulted in scaffolds with average fiber diameters of 2.556 μm and average pore area of 70.06 μm^2 , signifying the first attempt to characterize pore size of electrospun scaffolds in the BVM Lab. Additionally, Deven found that scaffolds produced with the BBB protocol were significantly different in fiber diameter, pore area, and wall thickness¹¹⁴.

1.6.5 Toni Pipes, 2014

The work conducted by Toni Pipes was the most recently published BVM thesis related to optimizing the electrospinning process within the lab. Toni tested several experimental electrospinning protocols along with the standard procedure developed by Tiffany Peña to investigate the effect of flow rate and applied voltage on mean fiber diameter. It was determined that the standard protocol elicited the most consistent fiber diameter results, and was used in a larger reproducibility study. This study suggested that the current electrospinning protocol did not create scaffolds with reproducible mean fiber diameter or mechanical compliance, citing the possibility that environmental conditions may significantly impact electrospinning results. The average fiber diameter of scaffolds fabricated for the reproducibility study was $2.22\text{ }\mu\text{m}^6$.

1.7 Summary and Aims of this Thesis

The in-house electrospinning vascular scaffold fabrication technique is critical to all work done in the Cal Poly BVM lab, and BVM set up and device testing cannot occur without it. The reliability and consistency of these scaffolds should then be of paramount concern. This thesis describes 5 aims undertaken to progress towards a more functional, reliable, and consistent scaffold fabrication procedure and to expand the capabilities of the BVM lab by fabricating scaffolds that more closely resemble the morphology and functional properties of native blood vessels. These 5 aims included:

- 1) Improve scaffold characterization by comparing two techniques for fiber diameter measurement and implementing a technique for pore area measurement.

The primary scaffold evaluation technique, measuring average fiber diameter, was compared to that of a potential replacement technique using several criteria to determine the most accurate and consistent technique with which the BVM lab will move forward. A secondary measurement technique designed to characterize scaffold pore area was also developed and described to provide a more complete approach to evaluating electrospun scaffold and drawing conclusions from their performance.

- 2) Reduce scaffold fiber diameter and pore area by investigating humidity and solvent composition as electrospinning parameters.

Relative humidity and solvent composition were investigated to reduce fiber diameter and pore area to more closely replicate conditions favorable to cell adhesion onto the scaffold's luminal surface. Previous attempts have been made within the BVM lab to achieve improved scaffold efficacy and they have focused mainly on the interplay between several electrospinning process parameters. The work described in this thesis includes consideration of these previous experiments as a foundation and expands upon them by exploring other factors previously untested.

- 3) Reduce process variability by developing a less ambiguous electrospinning protocol.

An overhaul of the basic solution mixing and electrospinning process was conducted to decrease variability by more precisely and explicitly describing key techniques and concepts involved in each process. This involved defining and consolidating values and techniques that previously only existed by word-of-mouth communication and varied across electrospinning users.

- 4) Improve scaffold consistency and use by understanding and reducing PLGA scaffold shrinkage.

One of the main issues regarding the use of the scaffolds was addressed, the matter of unpredictable scaffold shrinkage when exposed to standard BVM sterilization techniques and/or to incubation conditions. Several scaffolds were measured for dimensional changes after exposure to various standard BVM conditions and compared to scaffolds that had been previously treated in ways to specifically mitigate shrinkage while retaining the original desired scaffold dimensions.

- 5) Identify and evaluate more flexible polymers as potential alternatives for electrospun BVM scaffolds.

Alternative materials to PLGA for scaffold fabrication via electrospinning were evaluated to serve the needs of the BVM lab more appropriately. The selected

polymer systems were spun and evaluated based on their ability to form a scaffold with the current electrospinning protocol, fiber diameter and pore area, and minimum bend radius. The characterization described herein is intended to build the foundation for further investigation into the effects of materials selection and processing parameters on scaffold performance in the context of the BVM lab.

Work performed towards each of these five aims will be presented in the following five chapters, beginning with fiber diameter and pore size characterization techniques.

2. COMPARISON OF SCAFFOLD MEASUREMENT METHODS AND IMPLEMENTATION OF AN ADDITIONAL CHARACTERIZATION TECHNIQUE

2.1 Introduction

The first aim of this thesis was to improve BVM scaffold characterization by comparing two techniques for fiber diameter measurement and by implementing a new technique for pore area measurement. This involved finding a new fiber diameter measurement method that could be readily integrated into standard lab practices and could be directly compared to the current method used by BVM lab members. Additionally, the different ways in which the pore geometry of porous scaffolds effects cell behavior and how it is typically characterized was reviewed; this was done for the purpose of developing a pore area characterization technique for scaffolds produced in the BVM lab, and is described more completely in the following sections.

2.1.1 Fiber Diameter

There is a large body of research that suggests a correlation between fiber diameter of fibrous polymeric scaffolds and cell adhesion in tissue engineered constructs³¹⁻³⁴. Among these, Kwon et al. reported that human umbilical vein endothelial cells (HUVECs) adhered to electrospun 50:50 PLCL scaffolds of 0.3 and 1.1 μm mean fiber diameter significantly better than to scaffolds with 7 μm fiber diameter³¹. Similarly, Whited et al. reported significantly higher HUVEC coverage on 50:50 PCL-collagen scaffolds of 100 and 300 nm fiber diameters compared to scaffolds with mean fiber

diameter of 1200 nm³². Because of this, fiber diameter has been the main metric by which electrospun scaffolds in the BVM are assessed. However, there are concerns with the overall accuracy, reproducibility, and comparability to published literature results that call into question the efficacy of the current fiber diameter measuring method.

The current method by which members of the BVM lab measure electrospun scaffold fiber diameter involves using the publicly available image processing program ImageJ (Appendix A). SEM images of the luminal surface of a scaffold are taken and then bars are drawn manually across a subset of fibers within ImageJ⁶. The program measures the number of pixels encapsulated in this distance and converts this value into units of length using the scale bar of the image as a reference. Fibers to be measured are selected by overlaying either a 3x3 or 4x4 grid of circles onto the image, and the user is responsible for selecting the fiber nearest to the center of each circle that is wholly visible, typically the fibers closest to the lumen. This method is time consuming, as each image requires 16 separate measurements after manual manipulation to enhance contrast and overlay the circle template. It is also subject to potential variation across users and across measuring session by the same user based on the selection criteria for choosing a fiber to measure within a circle. Finally, there has been no prior investigation into how well the results of this method accurately represent the actual mean fiber diameter of the entire image. Ideally these concerns could be obviated by a standardized, more automatic method that measured most of the fibers within an image with less qualitative input from the user.

DiameterJ is a free-to-use plugin for ImageJ specifically designed for use with SEM micrographs of nanoscale, fibrous scaffolds¹¹⁵. It operates by binarizing an image through a process called segmentation. DiameterJ identifies fibers by locating their centerlines and correcting for instances of overlap or intersection (Figure 11). From this, a mean fiber diameter measurement is produced.

Figure 11. Detailed flow chart of the DiameterJ measuring technique and output¹¹⁵.

The possibility of measuring all the fibers within an SEM image is attractive, and the validating study showed no significant difference between DiameterJ and manual measurement fiber diameter results. However, while the authors did use PLGA nanofibers as a reference, the images used to validate the software plugin appear

somewhat different compared to those of electrospun PLGA scaffolds from the BVM lab, and thus the plugin may interpret them differently.

A portion of Aim I of this thesis was dedicated to comparing the results of the current manual fiber diameter measuring method and DiameterJ to determine which method would be most accurate, consistent, and easy to use. Ideally, this method will be robust enough to reduce or eliminate variability between operators and thus can also be used to compare data from within the BVM lab across several years.

2.1.2 Pore Area

In addition to fiber diameter, which has been a key metric in the BVM lab for years, pore area is also an important scaffold trait but has been largely neglected in the lab during typical scaffold characterization.

During the setup of some recent blood vessel mimics there have been observations of what appear to be cells passing transmurally through the pores of the scaffold during sodding. Multiple sources suggest that the largest dimension of SMCs and ECs is typically found to be approximately 96-139 μm , and it stands to reason that pores much larger than these dimensions may allow cells to pass through^{35,36}. In addition to these instances, there are several sources of tissue engineering research that suggest a strong correlation between pore size and cell coverage, similar that between fiber

diameter and cell coverage. Lee et al. tested the effect of pore diameter range on smooth muscle cell adhesion and growth. It was determined that 50-100 μm diameter pores had significantly higher cell coverage compared to with 100-150 and 150-200 μm diameters at 1, 7, and 14 days post-seeding onto solvent cast 85:15 PLGA scaffolds⁵¹. Similar results have been found with 3T3 mouse fibroblasts: O'Brien et al. reported that cell attachment rates to a porous collagen-glycosaminoglycan scaffold were highest in samples with pore diameters of 95.9 μm compared to those with pore diameters of 109.5, 121, and 150.5 μm ³⁸. Additionally, multiple studies showed favorable smooth muscle cell adhesion results with pore diameters in a 38-160 μm range on PLA scaffolds^{39,40}. However, presently there have only been incomplete attempts within the BVM lab to characterize this scaffold property¹¹⁴. **Thus, another aspect of Aim I of this thesis was dedicated to identifying and characterizing multiple pore size measurement methods and developing a lab protocol for future pore size measurements within the lab.**

In addition to manual distance measuring tools, ImageJ possesses the ability to measure areas of pixels automatically based on their intensity or place along the black-to-white spectrum. To make an image compatible with this method, one must use a thresholding tool to turn all pixels above or below a desired intensity into a solid color. ImageJ identifies this color and can mark and measure discrete areas formed by regions of this color. In this way, pores can be selected and measured for total area within a scaffold SEM image based on their intensity relative to that of the fibers (Figure 12).

Figure 12. SEM image of a PLGA scaffold (left), and the same image after thresholding (right)

Both DiameterJ and the fully manual method takes advantage of the native ImageJ Analyze Particles tool in an identical manner, however the former is done after image segmentation. Thus, comparing pore area results between a manual method and DiameterJ will effectively be comparing manual thresholding to DiameterJ segmentation techniques.

In addition to these direct measurements of pore size, pore area can be assessed with indirect methods such as capillary flow porometry. Capillary porometry consists of placing a scaffold sample into a sealed chamber across a gas entrance port, contacting it with a wetting fluid that fills its pores, and introducing a pressurizing gas^{116,117}. The gas pressure is increased until bubbles form and subsequently until the sample is completely dried. Using results from this test and information about the interaction between the fluid and the sample one can calculate pore diameter using the Washburn equation (1).

$$D = (4\gamma\cos\theta)/p \quad (1)$$

in which D is the diameter of a pore assumed to be cylindrical, γ and θ are the surface tension and contact angle of the wetting fluid on the scaffold material, respectively, and p is the pressure differential of the gas across the sample. Compared to direct measurements of SEM images taken of a porous scaffold, capillary flow porometry requires dedicated equipment specifically for pore size measurements. This equipment would need to be assembled, calibrated, and standardized within the lab, in addition to the other measurements required to determine values like surface tension and contact angle for a given fluid-scaffold material combination. Even with the assumption that these values could be obtained accurately there may be some deviation from “true” pore size values due to the assumption that all pores are of a cylindrical geometry. Because this is an indirect measurement method there may be some question as to its comparability with literature results that were determined via a direct image measurement technique¹¹⁶. Finally, the main concern of the BVM lab regarding pore size is with those present on the luminal surface of the scaffolds, as these pores are in direct contact with cells. Any gradients in pore size between the luminal and outermost surface of the scaffold will be obscured by the single-value mean pore size result of flow porometry method. For these reasons, the only pore size measuring techniques tested in this thesis were performed manually, using SEM images produced from a previously-established protocol.

Monitoring PLGA scaffold pore area within the lab over time and across multiple operators can aid in quickly assessing concerning trends that may be related to failing equipment or inappropriate scaffold fabrication techniques. The goal of developing a universal, easy-to-use pore size measurement technique will allow for comparison

between scaffolds fabricated by multiple operators and with previous data generated in the BVM lab. Additionally, this method will ideally provide the ability to accurately and consistently compare pores between BVM lab results and results from published literature. This can eliminate a source of uncertainty from the scaffold when comparing BVM lab-produced vessel constructs to those from other sources.

For the work in this Aim, ImageJ was used to assess the pore area using SEM images of several electrospun PLGA scaffolds produced for the purposes of this thesis, images from previous BVM lab theses, and images from previous electrospinning literature. A fully manual method of pore area measurements was developed using native ImageJ tools and was compared against the Mesh Hole Analysis tool within the DiameterJ plugin to evaluate accuracy, consistency, and ease of use.

2.2 Fiber Diameter Measurement Methods

The manual and DiameterJ fiber diameter measurement methods were compared on 3 bases: 1) overall accuracy against a control sample of known size, 2) consistency within an experimental sample at varying magnifications, and 3) comparability to other measurement techniques for electrospun scaffolds using images and data found in published literature. These methods were also compared directly using the experimental sample images to evaluate their differences in application.

The control sample used was a woven 316L stainless steel mesh with a known fiber diameter of 0.0012 inches or 30.5 μm (Figure 13)¹¹⁸.

Figure 13. Woven 316L stainless steel mesh used as a reference sample for fiber diameter and pore area measurements.

Experimental sample images were taken of 15wt% PLGA scaffolds at 600x, 800x, and 1000x for the purposes of this study, and additional images from past theses were also measured to compare results to those from other users of the manual measuring method. Because of the significant difference in size between the fibers of the metal mesh and the fibers of the scaffold, images of the mesh were taken at magnifications that presented metal fiber diameters at roughly the same pixel length as a polymeric fiber at 600x-1000x. This was done to most accurately simulate the conditions these methods will be conducted in for lab use. Additionally, because the contrast of images analyzed using the manual measuring technique are often altered after imaging, the difference in contrast

enhancement levels was characterized as well. Furthermore, this idea was expanded to include the DiameterJ method and investigate whether a post-processing enhancement of contrast affected the results of segmentation in any way. These methods will be described in detail below.

2.2.1 Manual Method

The manual fiber diameter measurement method began by enhancing the contrast of images taken directly from a Hitachi TM-1000 scanning electron microscope. Enhancement was performed in ImageJ by increasing the number of saturated pixels to 15% of those present in the image, as this appeared to be the threshold at which a large difference in intensity between fibers and voids became clear, yet little to no fiber detail was lost (Figure 14).

Figure 14. SEM images with increasing amounts post-processing contrast enhancement. Top is as-imaged, all other images represent increasing amounts of pixel saturation.

A 4x4 circle template was overlaid onto each image (circle diameter ~90 pixels), and the scale bars of representative images from each magnification were measured. The conversion between pixels and microns was noted and used for future measuring sessions to eliminate the need to manually measure a scale bar every time (Table I).

Table 1. Pixel-to-micron conversion ratio for SEM images

Magnification	80x	120x	200x	600x	800x	1000x
Scale Bar Length (μm)	1000	500	500	100	100	100
Pixels/micron	0.620	0.93	1.55	4.66	6.20	7.76

16 fiber diameter measurements were made per image by selecting the fiber closest to the middle of each circle that was fully visible, such that one could confidently use the Line tool in ImageJ to draw an uninterrupted line spanning the fiber perpendicular to its length direction (Figure 15).

Figure 15. Sample PLGA scaffold SEM image after manual ImageJ measurement, showing a 3x3 circle template with 1 fiber diameter measurement per circle.

The entirety of the manual measurement method for determining the fiber diameter of scaffolds imaged via SEM is described in Appendix A.

2.2.2 DiameterJ Method

The DiameterJ plugin required binary, black and white SEM images to properly assess fiber dimensions, and so this method began by selecting the available binarization algorithms with which to process the SEM images of interest. Initial attempts were made to isolate the most reliable algorithms for the sake of maintaining consistency across all measurements, however the most accurate algorithm did not remain constant from image to image. The most suitable segmented images were selected based the ability of the algorithm to show a representative sample of fibers that appeared to be of unaltered diameter, evaluated qualitatively. The most accurate segmented image(s) were then evaluated by the DiameterJ measuring process, as detailed in Figure 11. Unlike the manual method, DiameterJ generates a measurement for all fibers identified in the segmented image and thus uses a much larger sample size. This procedure is described in full in Appendix B.

2.2.3 Comparisons

4 tests were devised to characterize the differences between the manual and DiameterJ fiber diameter measurement methods:

Contrast Test: PLGA scaffolds were not sputter-coated prior to SEM imaging, and thus raw images were difficult to discern without increasing contrast as a post-processing technique. The first experiment compared 4 contrast levels using the native ImageJ “Enhance Contrast” tool to determine the point at which enhancing image contrast resulted in detail lost from the image, and to what degree a potential loss would have on each method.

Reference Mesh Test: A stainless steel mesh of known fiber and pore size was imaged and measured with both methods using identical techniques as typical PLGA scaffolds (Appendices B and C). This was performed to evaluate the absolute accuracy of each measurement method of a fibrous material. Each method was performed on images of 3 different magnifications to evaluate any variances as relative fiber size increased.

BVM Lab Images: Images of PLGA scaffolds produced in the BVM lab for the purposes of this thesis as well as images from past thesis were measured with both techniques. This test was a direct comparison between methods to evaluate general performance and variability using images equivalent to those regularly generated in the BVM lab. Images from past theses were compared to their reported value from their respective works.

Additionally, the images generated during the execution of this thesis were measured at three different magnifications to evaluate any variances between methods at increasing relative fiber size.

Literature Images: Finally, both methods were used to measure three images extracted from other published electrospinning literature. This was performed as a comparison between BVM lab methods and those used in literature to determine how accurate comparisons between BVM lab and outside literature can be.

2.3 Pore Area Measurement Methods

To assess the ability of a pore size measuring technique, 4 tests were devised similar to those executed when experimenting with fiber diameter measuring methods. First, each technique was performed on SEM images of 15wt% PLGA scaffolds (the BVM lab standard), either spun for the purposes of this experiment or repurposed from a previous thesis⁶. Images of these scaffolds at 600x, 800x, and 1000x were measured with each technique. Second, each technique was used on the stainless steel mesh control sample referred to previously, which has rectangular pores of $1.69 \times 10^{-6} \text{ in}^2$ or $1089 \text{ } \mu\text{m}^2$ area (0.0013 in. or 33 μm known side length). Third, these techniques were used to measure images from various literature sources to determine the comparability between methods available to the BVM lab and those in published research (Table II).

Table 2. Pore Area Measurement Image Descriptions

Image	Magnification(s)	Image Source	Reported Pore Size (μm^2)
Contrast_1	600x	BVM Lab	-
SS Mesh	80x, 120x, 200x	BVM Lab	1089
PLGA_1	600x, 800x, 1000x	BVM Lab	-
PLGA_2	600x, 800x, 1000x	BVM Lab	-
1T	500x	Toni Pipes ⁶	-
1D	1000x	Deven Patel ¹¹⁴	59.83
Lit_1P	-	Lowery et al. ⁵²	1164.16
Lit_2P	-	Rajzer et al. ¹¹⁹	21.36
Lit_3P	-	Rajzer et al. ¹¹⁹	1.19

2.3.1 Manual Image Thresholding

The fully manual method began with an unaltered SEM image in ImageJ. The contrast of this image was enhanced to 15% pixel saturation as described previously, then the thresholding tool was used to standardize the color of all pixels below a particular value on the 8-bit color scale (Figure 16).

Figure 16. Original SEM image of PLGA scaffold (top left). Contrast-enhanced SEM image (top right).

Insufficient thresholding limit that improperly highlights effective pore region (bottom left). Image thresholded to an appropriate amount (bottom right).

While altering image contrast to a fixed value aids in normalizing pixel shade across images from different sources or SEM sessions, thresholding values can be altered slightly to accommodate for any lingering differences on an image-to-image basis.

To obtain accurate pore selection results, the scale bar region of the SEM image was cropped, either before or after thresholding. Using the Analyze Particles tool, ImageJ detected the monochromatic regions of the image post-thresholding and determines their area by counting pixels enclosed within the regions' boundaries. This process can be refined by selecting a reasonable minimum and maximum region size detection limit and by requiring each area to have a certain circularity to be included. Both tools are used to eliminate “noise” in the results and attempt to focus on pores that are reasonable for cell adhesion (Figure 17).

Figure 17. A. Representative 15wt% PLGA scaffold SEM image at 600x magnification showing instances of improper pore detection (circled). B. SEM image after manual thresholding. C. Pore outlines produced from Analyze Particle ImageJ with no changes to min./max. particle size or circularity. D. Pore outlines at 0.1 – 1.0 circularity. E. Pore outlines identified with a minimum pore area of 200 pixels² (~10 μm^2). F. Pore outlines at 0.1 – 1.0 circularity and with a minimum pore area of 200 pixels².

Multiple iterations of these particle analysis parameters were tested and reported. Specifically, results were reported using multiple minimum pore area settings in an attempt to eliminate artificial lowering of pore area measurements due to inaccurate thresholding. Additionally, all results were generated with the ImageJ-provided option of excluding pores located at the edges of the image to eliminate the effect of a partial pore measurement on the overall mean pore size. The protocol for manual pore area measurements is also included in Appendix C.

2.3.2 DiameterJ Segmentation and Mesh Hole Analysis

DiameterJ pore area measurements were taken directly from DiameterJ Mesh Hole Analysis outputs generated for fiber diameter measurement trials. Like the manual method, the native ImageJ Analyze Particles tool is used with the broadest minimum pore area and circularity parameters set. Because of this, the main difference between the two methods is the use of an original SEM image for manual thresholding or the use of a segmented image processed through DiameterJ.

2.3.3 Comparisons

4 tests similar to those used to evaluate fiber diameter measurement methods were executed to investigate the differences between manual thresholding and the DiameterJ pore area measurement output.

Contrast Test: The same images and contrast levels (0, 5, 15, 40, and 80%) were measured with both pore area measurement techniques to determine if contrast level resulted in image deterioration and if this impacted pore area measurements.

Reference Mesh Test: The same stainless steel mesh of known fiber and pore dimensions was imaged and measured at 80x, 120x, and 200x to determine the overall accuracy of each method. Prior to this, however, the manual thresholding method was used on 80x, 120x, and 200x images of the reference mesh at increasing values for the minimum pore size detected by the ImageJ Analyze Particles command. Manual thresholding produces artifacts that will appear as pores but are simply small, dark areas of fibers and don't accurately represent actual pores; defining a minimum pore size will eliminate some or all artifacts and return a more accurate average pore size.

BVM Lab Images: The same images of PLGA scaffolds produced in the BVM lab were also measured with both pore area techniques as a direct comparison using images similar to those that would be used in regular application in the BVM lab to assess general performance and variability. Because pore area of scaffold images had not been measured regularly prior to this thesis, only image 1D had a known value with which to compare experimental results.

Literature Images: Finally, both methods were used to measure three images from other sources of electrospinning literature. This was performed as a comparison between the experimental methods considered in this thesis with those typically used in literature to assess how accurate direct comparisons of results will be in the future.

2.4 Fiber Diameter Results

All images were measured in accordance with Appendices A and B for manual and DiameterJ fiber diameter measurements. All statistical comparisons between experimental groups was done with a general linear model and Tukey pairwise comparisons while all comparisons to known values was done with a one-sample t-test with a hypothesis of $\mu \neq \mu_0$.

2.4.1 Contrast Test

A representative SEM image of electrospun PLGA scaffold spun for the purposes of this thesis was subjected to 4 separate contrast enhancements through ImageJ and was measured with both techniques to observe any differences created by the image alteration. It was made clear through comparing composite images of all possible segmentation options for a given SEM image called “montage images” that the contrast enhancement had no effect on the DiameterJ segmentation method and thus no effect on the fiber diameter measurement results (Figure 18 and 19). The images of varying levels of contrast enhancement were each measured with the manual method (Table III).

Figure 18. Montage images showing all segmentation options for 0% contrast enhancement (top) and 80% contrast enhancement (bottom), with the image before segmentation located in the top left corner of each montage.

Figure 19. Best segmentation option for 0% contrast enhancement (left) and 80% contrast enhancement (right); the images are identical, showing contrast does not affect segmentation.

Table 3. Fiber Diameter Contrast Test Results

Contrast Enhancement (%)	Manual Method		DiameterJ Output	
	Fiber Diameter (μm)	Std. Dev. (μm)	Fiber Diameter (μm)	Std. Dev. (μm)
0	4.58	1.09	3.33	1.02
5	4.58	0.99	3.33	1.02
15	4.66	1.07	3.33	1.02
40	4.58	1.09	3.33	1.02
80	4.17	1.06	3.33	1.02

Using a general linear model with Tukey pairwise comparisons it was determined that there was no significant difference between any of the manual method groups. Therefore, it was not shown that contrast level had an effect on the accuracy of the manual measurement method. As such, 15% contrast enhancement was used on all measured images presented in this thesis. DiameterJ output results were shown to be significantly different than all manual method groups except for 80% contrast enhancement.

2.4.2 Reference Mesh Test

The reference stainless steel mesh with known fiber size $30.5\ \mu\text{m}$ was measured using both techniques at 80x, 120x, and 200x to simulate the relative fiber-to-viewing area of PLGA scaffold images at high magnifications (Figure 20, Table IV).

Figure 20. Sample SEM images of the stainless steel reference mesh at 80x (top) and 200x (top) along with segmented versions for DiameterJ evaluation.

Table 4. Reference Mesh Fiber Diameter Results

Magnification	Manual Method		DiameterJ Output	
	Fiber Diameter (μm)	Std. Dev. (μm)	Fiber Diameter (μm)	Std. Dev. (μm)
80x	32.13	1.27	30.74	2.15
120x	30.52	0.54	29.97	1.59
200x	30.76	0.86	29.41	1.31

Figure 21. Boxplot of fiber diameter reference mesh results. *, $p < 0.05$ between groups. ***, $p < 0.001$ between groups.

Using a one-sample t-test it was shown that all method-magnification combinations had means significantly different from the known reference mesh fiber diameter value of $30.5 \mu\text{m}$ except for the manual method at 120x and 200x.

2.4.3 BVM Lab Images

A series of images produced within the BVM lab was then measured with each method to evaluate consistency and accuracy in application. This included images produced for the purposes of this study (PLGA_1 and PLGA_2) and images of known fiber diameter from previous BVM studies (1T and 1D) (Figure 22, Table V).

Figure 22. PLGA scaffolds from the BVM lab spun for this thesis (1,2), spun for Toni Pipe's thesis (3) and for Deven Patel's thesis (4)^{6,114}.

Table 5. BVM Image Fiber Diameter Results

Image Source	Magnification	Manual Method		DiameterJ Output		Reported Value	
		Fiber Diameter	Std. Dev.	Fiber Diameter	Std. Dev.	Fiber Diameter	Std. Dev.
		r (μm)	(μm)	r (μm)	(μm)	r (μm)	(μm)
PLGA_1	x600	4.29	1.00	3.33	1.02	-	-
PLGA_2	x600	3.79	1.49	2.71	1.26	-	-
1T	x500	1.91	0.58	1.80	0.56	1.71	0.37
1D	x600	3.60	1.04	3.04	1.02	2.52	-

It was determined that the results for PLGA_1 and PLGA_2 both did not significantly differ between methods using a general linear model with Tukey pairwise comparisons (Figure 23).

Figure 23. Boxplot of experimental fiber diameter values for PLGA_1 (top) and PLGA_2 (bottom).

Additionally, through use of a one-sample t-test it was observed that the manual method was not different from the known fiber diameter value of 1T, while the DiameterJ result was significantly different (Figure 24). The means of both methods were significantly different than the known value of 1D, observed through use of a one-sample t-test as well (Figure 25).

Figure 24. Boxplot of experimental fiber diameter values for sample 1T. \$ signifies difference compared to known value of 1.71 μm , $p < 0.05$.

Figure 25. Boxplot of experimental fiber diameter values for sample 1D. \$ signifies difference compared to known value of 2.52 μm , $p < 0.05$. *, $p < 0.05$ between groups.

2.4.3.1 Magnification Test

Images PLGA_1 and PLGA_2 were then measured at 800x and 1000x as well to evaluate any differences between 600x magnification results for each method (Figure 26 and Table VI).

Figure 26. SEM and segmented images of PLGA_1 at 600x (top), 800x (middle), and 1000x (bottom).

Table 6. Fiber Diameter Results for Magnification Test

Image Source	Magnification	Manual Method		DiameterJ Output	
		Fiber Diameter (μm)	Std. Dev. (μm)	Fiber Diameter (μm)	Std. Dev. (μm)
PLGA_1	600x	4.29	1.00	3.33	1.02
	800x	4.06	0.58	3.13	1.06
	1000x	3.92	0.70	3.18	1.14
PLGA_2	600x	3.79	1.49	2.71	1.26
	800x	2.91	1.37	2.33	1.05
	1000x	2.80	1.17	2.16	0.98

Fiber diameter results from the magnification test were subjected to a general linear model with Tukey pairwise comparisons, and it was determined that the DiameterJ method differed significantly between magnifications for both PLGA_1 and PLGA_2 measurements (Figure 27 and 28).

Figure 27. Boxplot comparison of PLGA_1 between measurement methods. # signifies difference from Manual, 600x, $p < 0.05$. ***, $p < 0.001$ between groups.

Figure 28. Boxplot comparison of PLGA_2 between measurement methods. # signifies difference from DiameterJ, 600x, $p < 0.05$. *, $p < 0.05$ and ***, $p < 0.001$ between groups.

2.4.4 Literature Images

In addition to images of PLGA scaffolds spun in the BVM lab, SEM images of electrospun fibers of various materials were also measured with both methods. This was performed to determine how reliable each method was at simulating the results of methods used by other researchers. Images were selected based on resolution and the relative fiber size to total size of the image to ensure that the circle template from the manual method would select a representative group of fibers. Each image was measured using the protocols outlined in Appendices A (Figure 29, Table VII).

Figure 29. SEM images for fiber diameter measurements from various literature sources of electrospun gelatin (top), PLGA/collagen copolymer (middle), and PLLA (top)^{49,120,121}.

Table 7. Fiber Diameter Results for Literature SEM Images

	Manual Method		DiameterJ Output		Reported Value	
	Mean (μm)	Std. Dev. (μm)	Mean (μm)	Std. Dev. (μm)	Mean (μm)	Std. Dev. (μm)
Lit_1F	0.662	0.052	0.478	0.260	0.600	-
Lit_2F	0.226	0.067	0.211	0.078	0.272	0.062
Lit_3F	1.534	0.364	1.189	0.525	1.25	-

The use of a one-sample t-test with a hypothesis of $\mu \neq \mu_0$ revealed that both the manual and DiameterJ measurement methods were significantly different from the reported values of each literature image (Figure 30).

Figure 30. Boxplots for experimental fiber diameter results for Lit_1F (top), Lit_2F (middle), and Lit_3F (bottom). \$ signifies difference from known value, $p < 0.05$. **, $p < 0.01$ between groups.

2.5 Pore Area Results

All images were measured in accordance with Appendices C and B for manual and DiameterJ pore area measurements, respectively. All statistical comparisons between experimental groups was done with a general linear model and pairwise comparisons, while all comparisons to known values was done with a one-sample t-test with a hypothesis of $\mu \neq \mu_0$. Additionally, all boxplots of pore area measurements were generated with outliers omitted using the interquartile range outlier rule^{122,123}. This was done to reduce the bias of overly large “pores” detected by ImageJ due to thresholding that connected several, more reasonably sized pores (Figure 31).

Figure 31. Example of a large area of an SEM image outline being marked as a single pore by manual ImageJ thresholding (top, marking in red) and its corresponding original image (bottom).

2.5.1 Contrast Test

Native ImageJ contrast enhancement was performed on all images used for pore area measurement for the purposes of this thesis. While it was shown that no significant difference existed between various contrast enhancement levels on the manual fiber diameter measurement method, the test was performed on the same image for pore area evaluation to ensure the same level of certainty (Table VIII). The DiameterJ Mesh Hole Analysis output was not considered for this experiment since it was previously shown that contrast enhancements have no impact on the DiameterJ segmentation process, however the pore area was provided for the sake of comparison. SEM images of increasing contrast enhancement treatments were presented earlier in Figure 14.

Table 8. Pore Area Contrast Test Results

Contrast Enhancement (%)	Manual Method		DiameterJ Output	
	Pore Area (μm^2)	Std. Dev. (μm^2)	Pore Area (μm^2)	Std. Dev. (μm^2)
0	238.58	154.69	93.27	94.08
5	160.75	91.00	93.27	94.08
15	299.91	189.68	93.27	94.08
40	339.80	287.66	93.27	94.08
80	299.91	189.68	93.27	94.08

All method-contrast level combinations were compared through use of a general linear model with Tukey pairwise comparisons; there existed no significant difference between mean of the manual method at 15, 40, and 80% and between 0, 15, and 80%, however all other comparisons did show significant differences (Figure 32).

Figure 32. Boxplot representing pore area results for the contrast test. #, $p < 0.05$ difference between all other groups. *, $p < 0.05$ difference between groups.

Because it was not shown to be significantly different than SEM images measured at 0% contrast enhancement, 15% pixel saturation was used on all measured images presented in this thesis. It was noted that the DiameterJ output results were shown to be significantly different than all manual method groups.

2.5.2 Reference Mesh Test

Measurements of the reference image were done for 2 reasons: 1) to help determine an appropriate minimum pore area for the ImageJ Analyze Particles tool with which to move forward, and 2) to compare the total accuracy of each pore area measurement method by using a sample of known pore area.

2.5.2.1 Minimum Pore Area Restriction Test

The reference mesh was measured at 80x, 120x, and 200x as these magnifications best mimicked the ratio of fiber diameter to total image size of the scaffold for the size of the mesh fibers. Each magnification was measured with increasing minimum pore area restrictions, beginning with no minimum restriction and ending with $150\ \mu\text{m}^2$. The average pore size of the reference mesh is $1089\ \mu\text{m}^2$, and each magnification test of the manual method achieved a result within 10% of this value by the $10\ \mu\text{m}^2$ minimum pore restriction (Figure 33 and 34, Table IX). While every pore of the reference mesh images was not identified properly, the clear majority of identified areas appeared to be actual pores of the mesh. Additionally, as the minimum pore area increases the instances of incorrectly identified areas reduces noticeably (most evident in the lower right corner of the outline images).

Figure 33. Thresholded images and outlines of pores for the minimum pore area restriction test at 80x magnification, with minimums of 0 μm^2 (top), 10 μm^2 (middle), and 50 μm^2 (bottom).

Figure 34. Pore area measurements of the reference mesh material at increasing minimum pore area restrictions for 80x, 120x, and 200x magnifications.

Table 9. Pore Area Values for Reference Mesh Minimum Pore Area Restriction Test

Magnification	Minimum Pore Area Restriction (μm^2)				
	0	10	50	100	150
80x	702.37	984.78	1031.97	1033.33	1034.58
120x	577.59	989.95	1013.31	1013.31	1013.31
200x	480.92	997.74	1014.68	1014.68	1014.68

At 80x, 120x, and 200x magnifications the minimum pore area restriction of 50 μm^2 , the point at which the trend of increasing pore area levels off, is equal to 19.22, 43.24, and 120.12 pixels², respectively. Because the 80x image produced pore area values closest to the known pore area value of the reference mesh, a minimum pore area restriction size of 19.22 pixels² will be used for all future manual pore area measurements to eliminate any thresholding artifacts in an effort to keep all results consistent with one another.

2.5.2.2 Direct Measurement Method Comparison

A direct comparison between the manual pore area measurement method and the DiameterJ Mesh Hole Analysis output was made using the reference mesh. By measuring the reference mesh at 80x, 120x, 200x each method was compared against the known average pore area value of $1089 \mu\text{m}^2$ (Table X). All manual method values are presented using the Analyze Particles tool with a new minimum pore area of 19.22 pixels^2 . Samples of the thresholded images from the manual method and segmented images from the DiameterJ output were provided in Figure 20, 33 and 34 and Table IX.

Table 10. Pore Area Values for Reference Mesh Method Comparison

Magnification	Manual Method		DiameterJ Output	
	Pore Area (μm^2)	Std. Dev. (μm^2)	Pore Area (μm^2)	Std. Dev. (μm^2)
80x	1031.97	312.76	879.24	163.73
120x	1015.12	92.084	946.60	89.01
200x	1028.23	74.78	1014.67	71.26

It was determined that all measured values of reference mesh pore area were significantly different than the known value. It was also observed that results of DiameterJ at 80x and 120x were significantly different than all other groups (Figure 35).

Figure 35. Boxplot of reference mesh pore area values for manual and DiameterJ methods at various magnifications. \$, $p < 0.05$ difference between known value of $1089 \mu\text{m}^2$. #, $p < 0.05$ difference between all other groups besides DiameterJ, 80x. @, $p < 0.001$ difference between all other groups.

2.5.3 BVM Lab Images

The same series of images from fiber diameter method comparison tests were used to compare pore area measurement methods as well. PLGA_1, PLGA_2, 1T, and 1D were measured as a direct comparison between methods, and 1D was compared against a reported pore area value published by Deven Patel in his thesis (Figure 36 and 37, Table XI).

Figure 36. BVM lab images with pore accompanying pore outlines of PLGA_1 (top) and PLGA_2 (bottom).

Figure 37. BVM lab images with pore accompanying pore outlines, of 1T (top) and 1D (bottom).

Table 11. BVM Image Pore Area Results

Image Source	Magnification	Manual Method		DiameterJ Output		Reported Value	
		Pore Area (μm^2)	Std. Dev. (μm^2)	Pore Area (μm^2)	Std. Dev. (μm^2)	Pore Area (μm^2)	Std. Dev. (μm^2)
PLGA_1	600x	171.54	142.32	97.72	98.84	-	-
PLGA_2	600x	225.63	193.98	75.08	75.52	-	-
1T	500x	43.13	53.24	93.48	214.56	-	-
1D	1000x	16.19	28.34	9.76	12.63	59.83	-

It was determined that the manual and DiameterJ methods produced significantly different means when measuring PLGA_1, PLGA_2, and 1T (Figure 38).

Figure 38. Boxplots of experimental pore area values for PLGA_1 (top), PLGA_2 (middle), and 1T (bottom). **, p<0.01 difference between groups. ***, p<0.001 difference between groups.

Additionally, a one-sample t-test was used on 1D data to compare the experimental pore area measurement method results with the mean pore area published by Deven. It was determined that both the manual and DiameterJ methods were significantly different than the published mean area; additionally, a general linear model with Tukey comparisons showed that the experimental means were not different from each other (Figure 39).

Figure 39. Boxplot of experimental pore area values for sample 1D. \$, $p < 0.05$ difference from published value of $59.83 \mu\text{m}^2$.

Next, PLGA_1 and PLGA_2 were measured at increasing magnifications to observe any variation in results of either method as the fiber size-to-image size ratio increased.

2.5.3.1 Magnification Test

As in the fiber diameter measurement methods experiments, a test investigating the differences in feature measurement with image magnification was conducted with

pore area as well. PLGA_1 and PLGA_2 were measured at 600x, 800x, and 1000x with both the manual pore area method and DiameterJ Mesh Hole Analysis and compared directly to observe any differences (Table XII).

Table 12. Pore Area Results for Magnification Test

Image Source	Magnification	Manual Method		DiameterJ Output	
		Pore Area (μm^2)	Std. Dev. (μm^2)	Pore Area (μm^2)	Std. Dev. (μm^2)
PLGA_1	600x	171.54	142.32	97.72	98.84
	800x	117.21	113.49	91.51	103.62
	1000x	62.72	73.92	49.86	50.59
PLGA_2	600x	225.63	193.98	75.08	75.52
	800x	216.64	221.02	90.35	87.92
	1000x	156.84	148.65	74.35	93.24

Using Tukey pairwise comparisons it was determined that no significant differences existed between any manual measuring methods, however it was observed that differences existed between DiameterJ and the manual method results at 600x magnification (Figure 39).

Figure 39. Boxplot of PLGA_1 pore area measurements at 600x, 800x, and 1000x. #, $p < 0.01$ difference between Manual 600x. @, $p < 0.001$ difference between Manual 600x.

A similar observation was made regarding the pore area results of PLGA_2, however there was also a significant difference between values produced by the manual method at 600x and 1000x (Figure 40).

Figure 40. Boxplot of PLGA_2 pore area measurements at 600x, 800x, and 1000x. \$, $p < 0.05$ difference between Manual 600x. #, $p < 0.001$ difference between Manual 600x and 800x. @, $p < 0.05$ difference between Manual 1000x.

Finally, both methods were used to measure multiple images of electrospun samples from published literature as preliminary investigation into the accuracy of comparisons between BVM lab results and those in outside research.

2.5.4 Literature Images

Literature images from various published electrospinning research of PCL/PEO, gelatin, and PCL were measured with both methods to compare them directly to a reported literature value (Figure 41 and Table XIII).

Figure 41. SEM images for pore area measurements from various literature sources of electrospun PCL/PEO (top), gelatin (bottom left), and PCL (bottom right)^{52,119}.

Table 13. Pore Area Results for Literature SEM Images

	Manual Method		DiameterJ Output		Reported Value	
	Mean (μm^2)	Std. Dev. (μm^2)	Mean (μm^2)	Std. Dev. (μm^2)	Mean (μm^2)	Std. Dev. (μm^2)
Lit_1P	117.14	148.53	109.16	160.63	1164.16	-
Lit_2P	41.90	31.20	80.81	89.70	21.36	-
Lit_3P	21.96	16.00	33.58	54.11	1.19	-

Through use of a one-sample t-test it was determined that both the manual and DiameterJ pore area measurement methods produced mean values that were significantly different than the respective published values for all three images from literature. Additionally, the results of both experimental methods differed significantly when measuring Lit_2P and Lit_3P (Figure 42).

Figure 42. Boxplots of experimental pore area results for Lit_1P (top), Lit_2P (middle), and Lit_3P (bottom). \$, $p < 0.05$ difference from known value. *, $p < 0.05$ between groups. **, $p < 0.01$ between groups.

2.6 Fiber Diameter Discussion

2.6.1 Contrast Test

To preserve time and resources, and because it is not absolutely necessary for the standard BVM lab scaffold characterization techniques, PLGA scaffolds are rarely sputter coated prior to SEM imaging. The relatively low conductivity of bare electrospun PLGA scaffolds results in SEM images with poor contrast, and thus discerning fibers and other features can be difficult. Because of this, the contrast of images is often increased as a post-processing step, however at high contrast enhancement levels it became clear that the edges of features become distorted, possibly influencing the measured fiber diameter. The contrast test was performed to investigate whether a significant difference existed between raw SEM images and those with enhanced contrast using the native ImageJ Enhance Contrast tool at 5, 15, 40, and 80% pixel saturation for either measurement method. Using a general linear model with Tukey pairwise comparisons it was observed that **no significant difference existed between an SEM image at any contrast level using the manual fiber diameter measurement method**. The DiameterJ segmentation method was also unaffected by the contrast enhancement, and the resulting fiber diameter was significantly different than all manual method measurement results except for that at 80% enhanced contrast. This suggests that contrast does not have a significant impact on measured fiber diameter, and so 15% was used for all remaining measurements presented in this thesis. These results also suggest that the manual method and DiameterJ output produce significantly different results, however this relationship was more conclusively explored with other tests.

2.6.2 Reference Mesh Test

Both measurement methods were used on a reference material, a piece of stainless steel wire mesh of known fiber diameter and pore size, to characterize their accuracy. Because the reference mesh fibers were approximately 10 times larger than electrospun PLGA, the SEM image magnifications used were 80x, 120x, and 200x. Using a one-sample t-test it was observed that the results of DiameterJ measurements at all magnifications (30.74 to 29.41 μm with ascending magnification) and of the 80x manual method measurement (32.13 μm) were significantly different than the known fiber diameter of 30.5 μm , while the 120x and 200x manual method measurements were determined to be statistically similar (30.52 and 30.76 μm , respectively). The reference mesh had fibers and pores of regular, predictable sizes and spacings, and thus is not perfectly analogous to a randomly oriented electrospun sample; However, **these results suggest that a higher accuracy is achieved at higher magnifications for the manual fiber diameter measuring method than at lower magnifications or with the DiameterJ method.** Because of this, SEM images were measured at 600x magnification for the duration of this thesis. Additionally, the standard deviations of the DiameterJ method results were 2-3 times larger than those of the manual method; Because all the fibers of the mesh are supposedly of equivalent size this suggests that DiameterJ measurements more frequently deviate from the given fiber diameter value on a per-measurement basis, yet still average to a result close to that of the given value.

While the most important trait of a scaffold characterization method for the BVM lab is reproducibility and comparability between scaffolds produced in the lab to identify

the source of BVM result trends, the findings of the reference mesh test suggest that the manual method may also produce values closer to the true diameter of the fibers measured; This may help establish confidence in measured BVM lab values when comparing electrospinning methods and results to those presented in literature.

2.6.3 BVM Lab Images

Next, both measurement methods were tested on a variety of SEM images of electrospun PLGA: two images, PLGA_1 and PLGA_2 were of scaffolds spun for the purposes of this thesis while 1T and 1D were extracted from prior BVM theses^{6,114}. These tests were performed to evaluate and compare each method using SEM image references directly analogous to those regularly produced in the BVM lab. Similar to the statistical analysis of the contrast test, a general linear model with Tukey pairwise comparisons was used on results from PLGA_1 and PLGA_2; It was determined that no significant differences existed between either method for either image. The same technique was used on 1T and 1D: The results of each method were not significantly different when measuring 1T, but did show significant difference when measuring 1D. These results suggest that the manual method and DiameterJ output produce results more similar to one another than may be assumed solely from the reference mesh test. It might be the case that the manual method is more accurate in measuring a reference mesh constructed of a highly regular pattern. However, the ability of a given method to produce reliable results with a randomly oriented, unpredictably sized fiber mesh is of greater importance. The methods still did differ when measuring 1D, however this image was of relatively low quality and may have suffered from a reduction in feature detail. These results suggest

that the manual and DiameterJ method do not produce results that differ significantly when measuring PLGA scaffolds spun in the BVM lab.

The fiber diameter value of 1T and 1D measured by the respective thesis authors was known, and so the manual and DiameterJ method results were also subject to a one-sample t-test: The test for 1T showed that the manual method was not significantly different than the mean fiber diameter measured by Toni Pipes for 1T, however the DiameterJ method result was. Both methods were found to be significantly different than the mean fiber diameter measured by Deven Patel for 1D. The lack of difference between the manual method and the published fiber diameter value of 1T along with the presence of significant difference between the manual method and known value for 1D suggest that the measurement technique utilized in this thesis may be more similar to that used by Toni than the one used by Deven. Deven used a circle template selection mask with 9 circles and thus 9 measurements per image, while this thesis and Toni's utilized a 16 circle template. This may indicate that Deven's measurements did not capture a representative sample fiber diameters, or that the criteria for selecting fibers within a circle was somehow biased towards fibers of a certain size. The fact that the DiameterJ method was not similar to either known value for 1T or 1D suggests that it does not produce results that are directly comparable to those from previous theses, and may present issues when comparing historical results to present data unless past images are all re-measured using DiameterJ. These results suggest that, while one form of statistical analysis showed that the measurement methods did not produce results that differed

significantly, the manual method was more accurate than DiameterJ in producing similar results to those of previous BVM lab image measurement techniques.

2.6.4 Literature Images

Finally, each measurement method was used on 3 SEM images retrieved from electrospinning literature from sources outside the BVM lab. This was done as a rudimentary comparison between measurement methods within and outside the BVM lab to evaluate how accurately results between sources could be compared. Images of gelatin, PLGA/collagen copolymer, and PLLA were imaged and compared to the known average fiber diameter value retrieved from their respective sources^{49,120,121}. Using a one-sample t-test, it was determined that both the manual and DiameterJ fiber diameter measuring methods were significantly different than the published value for each SEM image from literature. Unfortunately, the literature sources did not describe their respective fiber diameter measuring processes in detail, however it is possible that either the techniques used there or those described here did not obtain a representative sample of fibers. Because the raw SEM images were not available, image-capturing software was used to obtain the images, degrading the quality further from the already low-resolution published version. These images were also much smaller than those produced by the SEM used in the BVM lab, meaning that even if the images were of similar resolutions there would be less pixels per fiber and thus less fidelity and less measurement accuracy. These results suggest that both measurement techniques evaluated in this thesis are significantly different than those used in several literature sources, and thus BVM lab results cannot be directly compared.

2.6.5 General Method Comparison

The BVM lab has been using some form of the manual fiber diameter measurement method since 2009 when the electrospinning system was established⁷. However, in an attempt to reduce opportunities for operator variability and to decrease the time spent analyzing SEM images, a quicker, more automated fiber diameter measurement method was desired. DiameterJ was selected as a possible replacement for the manual method due to its relative simplicity and automation, ability to interface with ImageJ (software already used in the BVM lab), and the fact that it was developed specifically to measure tissue engineering scaffold fibers on the nanoscale¹¹⁵.

Independent from the quantitative results discussed in this section, there are multiple caveats to the idea that DiameterJ provides a rapid measuring alternative; DiameterJ requires images to be presented as binary black and white images before measuring can occur; This can be done through a partner plugin that performs a process called “Segmentation” on the image, automatically determining areas of black and white and converting the image accordingly. The segmentation process can generate between 8 and 24 possible options based on the number of algorithms the operator wishes to use, and the most accurate segmented image must be manually selected by the operator before measurements can begin. Ideally the algorithm used to obtain the most accurate option would remain constant for images taken with similar settings and of similar materials and morphologies (as most BVM lab PLGA scaffolds are), allowing the operator to simply look for the particular algorithm title used and selecting that image for measuring. However, the most accurate algorithm and resulting segmented image are not always the

same from scaffold to scaffold, and thus the operator must spend time comparing the details of each candidate segmented image to the original, slowing the process significantly. Additionally, the “most accurate” segmented image may change based on operator just like the current manual method (Figure 43).

Figure 43. Original SEM image with two representative segmented PLGA scaffold of similar segmentation; determining the most accurate is based on operator discretion.

Frequently there is no “perfect” segmentation option; some segmented images omit noticeable portions of the image while others incorporate more fibers but may have thickened all of them slightly, or may have produced small, unfinished fibers where darker background fibers exist on the original image. These will skew fiber diameter

measurements lower even though it appears that more fibers (and logically, a more robust sample) are being measured, for example (Figure 44).

Figure 44. Original SEM images overlaid with two representative segmented images showing several areas of imperfect segmentation.

Overall, these tests produced several important findings regarding fiber diameter evaluation:

- The contrast test showed that DiameterJ is completely unaffected by contrast, and that the manual method produces similar results regardless of contrast level.
- The reference mesh test showed that all DiameterJ method results differed significantly from the given value. The manual method produced results not significantly different than the given value above magnifications of 80x, suggesting that the manual method can produce diameter results similar to that of the true value of a fiber.
- Both measurement methods produced results not found to be significantly different from each other for several images taken in the BVM lab. However, the manual method was shown to be more accurate in matching the given values of images published in previous BVM lab theses, suggesting that DiameterJ would not be suitable for comparing current results to those of theses past.
- The literature image test showed that both methods tested herein were significantly different than those used in literature and thus BVM lab results cannot be compared directly through use of these methods.

2.7 Pore Area Discussion

2.7.1 Contrast Test

As with the fiber diameter contrast test, SEM images of PLGA scaffolds of several post-processed contrast enhancements were measured with both pore area measuring techniques to determine if any noticeable image deterioration occurred with increasing contrast enhancement. The results of the pore area contrast test were less unanimous than the fiber diameter test; using a general linear model with Tukey pairwise comparisons it was determined that contrast enhancements of 15, 40, and 80% as well as 0, 15, and 80% did not differ significantly within each group. 5% contrast and the DiameterJ output (which had been previously shown to not be influenced by contrast) were both significantly different than all other results. These results do not show a clear trend in pore area with increasing contrast level, suggesting that image deterioration is of little concern when measuring pore area with either method. However, this does not account for the significant reduction in measured pore area that occurred at 5%. When comparing the thresholded versions of the original and 5% images there appears to be little difference in the amount and location of pores, however the outlines generated by ImageJ show the difference more clearly (Figure 45). This suggests that minute differences in thresholding may have substantial consequences for the way in which pores are identified by ImageJ which is not promising for the manual pore area measurement method.

Figure 45. Manually thresholded (top) and outlined (bottom) SEM images at 0% (left) and 5% contrast enhancement). While the thresholded images appear relatively similar there are noticeable differences between the outline images generated by ImageJ.

Similar to the results of the fiber diameter contrast test, the difference between the DiameterJ Mesh Hole Analysis output and all results from the manual measurement method was an early indication that the two methods would consistently produce significantly different results for a given image; however, this relationship was more conclusively explored with other tests.

2.7.2 Reference Mesh Test

The reference stainless steel mesh sample was used for two experiments involving the pore area measurement technique. The first of these was performed to characterize and reduce the variability introduced by the Threshold and Analyze Particles tools native to ImageJ: manually thresholding an image causes smaller areas of darker pixels within a fiber to be counted as pores when using the Analyze Particles tool (Figure 46).

Figure 46. Representative thresholded SEM image of a PLGA scaffold (top) with accompanying pore outline from the ImageJ Analyze Particles tool (bottom). Specks on the outline image indicate small “pores” detected based on the thresholding process.

These artifacts did not represent actual pores within the scaffold and artificially lowered the average pore diameter. Fortunately, the Analyze Particles tool can be programed to only identify pores within a certain range, and so measurements of the reference mesh at increasing minimum identified pore area were made at 80x, 120x, and 200x magnification to find a point at which these artifacts were fully eliminated. Based on the shape of the trendline and the evaluation of results using Tukey pairwise comparisons it was determined that a minimum pore area of 19.22 pixels² was sufficient to eliminate enough artificial “pores” to negate their effect on pore area data. This minimum detected pore area value was used for all future manual pore area measurements.

Both pore area measurement methods were then used to measure SEM images of the reference mesh of known dimensions at 80x, 120x, and 200x magnification. Using a one-sample t-test it was determined that all of the manual or DiameterJ pore area results were significantly different than the known pore area of 1089 μm^2 . This suggests that neither method is entirely accurate at determining the absolute value of an average feature size, however they still may be useful for relative comparison between scaffold images within the lab. The potential variability and relative comparability of each method was tested further with SEM images of electrospun PLGA and other polymers in the following sections.

2.7.3 BVM Lab Images

Both pore area measuring methods were tested on SEM images of electrospun PLGA scaffolds produced in the BVM Lab, as stated in the fiber diameter measurement methods discussion. PLGA_1, PLGA_2, and 1T were used to directly compare the methods and either determine if they produce similar results or, if dissimilar results occur regularly, if a consistent difference between the two measurement methods existed. Image 1D was extracted for a previous BVM thesis in which Deven Patel attempted to characterize average pore size using ImageJ; his method included manually selecting a representative sample of pores from an SEM image using a 3x3 circle template and outlining them manually using the ImageJ “Freehand Selection” tool. The published average pore area value for 1D was compared to the experimental results produced in this thesis to determine how similar each method was to that which Deven used.

Using a general linear model with Tukey comparisons, it was determined that the two experimental pore area measurement methods produced means significantly different from each other when measuring images PLGA_1, PLGA_2, and 1T, and were similar when measuring 1D. Furthermore, through use of a one-sample t-test it was determined that both experimental means were significantly different than the published pore area value of 1D. These results of direct method comparison suggest that the manual and DiameterJ pore area measurement processes are significantly different. However, the t-test results of 1D do not necessarily suggest that both methods are inaccurate; the method used and results produced by Deven may not accurately represent the true average pore area of the image for one main reason: the size of the pore is completely dependent on the

operator's discretion (Figure 47). While the thickness of a fiber is clearly defined by its appearance, the bounds of a pore may be less apparent.

Figure 47. Example of pore area measurement presented in Deven's thesis¹¹⁴.

Figure 47 shows an image published in Deven's thesis that provides an example for how pores were measured with the method described. However, the selection of the pore was only regulated in its location within the image, with the use of a circle template. The size of the pore was not determined by anything quantifiable, simply by operator discretion which may vary widely across multiple users. For instance, an argument could be made for noticeably larger or smaller pores to be outlined that occupy some of the same space as the pore shown in Figure 48.

Figure 48. Examples of other pore areas (yellow) in place of that shown in Figure 47 (white) that may be considered equally valid pore selections.

While the specific combination of location, density, and spacing of fibers within a porous scaffold most conducive to cell adhesion are currently unknown, cell behavior can instead be correlated to some consistent, quantitative measure of pore size to achieve an understanding of their interaction sufficient for BVM lab use. The method presented by Deven does not achieve this because there is no guarantee that discretion in pore area selection will be similar across all measurements or by all operators. The use of a thresholding tool that consistently selects many pores simply based on relative pixel color comes much closer to achieving an indiscriminate, unbiased pore selection process. Because the ideal scenario for cell adhesion is not currently known, this pore measurement method may not produce the true average pore area value for a given image, however with thresholding and pore analyzing parameters held constant the results of several images may be compared in a relative manner more precisely than through use of other methods.

As with the fiber diameter measurement methods experiments, images PLGA_1 and PLGA_2 were also measured at 600x, 800x, and 1000x magnifications with both methods to investigate any possible variances. Through GLM with Tukey comparisons it was determined that for PLGA_1, measurements of all magnifications for a given method were similar, and only the 600x manual method results were different than any DiameterJ method results. Furthermore, average pore area decreased as magnification increased for both methods, and all manual method means were larger than all DiameterJ means. The trend of decreasing pore area with increasing magnification and the fact that the manual method produced higher average values than the DiameterJ method was observed with

PLGA_2 as well, the only exception being the fact that the 600x DiameterJ mean was the smallest average pore area overall. These results suggest a strong trend of the manual method with magnification. While this further complicated the issue of describing the parameters that would provide greatest overall accuracy with this method, it further reinforced the notion that a relative comparison may be achieved if parameters (including magnification) are held constant between images.

2.7.4 Literature Images

Finally, both pore area measurement techniques were performed on 3 images taken from electrospinning literature sources. Each result was compared to the published average pore size using a one-sample t-test; pore size was most often presented as a diameter in literature, and values were converted with a simple area calculation with the assumption that all pores were circular in nature. The t-test revealed that both manual and DiameterJ method measurements were significantly different than the published value for all 3 literature images. The pore sizes described in literature were obtained with either mercury porisometry or a capillary flow porosimeter, both of which generate an average pore value through calculations involving the pressure required to force a liquid through a porous material^{52,119}. These methods are well-suited for determining the overall porosity of a material, however the focus of the BVM lab pore area measurements are on the size of the pores on the luminal surface of the scaffold and their impact on cellular response.

A general linear model with Tukey comparisons also showed that the manual and DiameterJ measurement methods produced means significantly different from one another in 2 of the 3 images from literature. This is additional evidence that the two methods produce results consistently different from one another. Furthermore, there is no clear relationship between the two methods; the measurements of PLGA_1 and PLGA_2 showed that the manual method produced average pore areas consistently larger than those produced by the DiameterJ method, however measurements 2 of the 3 literature images showed the opposite to be true. In this way, it is clear that both methods cannot be used interchangeably to evaluate pore area in the BVM lab moving forward.

2.7.5 General Comparisons

Despite the name, the manual method seemed to automate the pore area selection process to a greater degree than the DiameterJ method. If an accurate set of parameters is agreed-upon for thresholding and analyzing pores, then the operator must do very little; most of the process can be automated using an ImageJ macro. The DiameterJ Mesh Hole Analysis process is identical to that of the fiber diameter measurement procedure in that operators must select the most accurate segmented image from a range of 8-24 possible segmentation algorithms. Furthermore, the most accurate algorithm changed from image to image, meaning the operator must determine the most accurate segmented image for each image to be measured. This is a tedious task that still introduces operator bias.

The manual pore area measurement method is not perfect, and the procedure described herein (Appendix C) is not complete by any means. The thresholding characteristics of an image change based on the processing that came before it, meaning that the specific path an image took to become a contrast enhanced, 8-bit binary, thresholded images alters the histogram of pixel saturation in different ways. Thus, a given threshold value (specifically the pixel color above/below which all other pixels will be counted) may change if steps are omitted or changed. In this way, results of a given image are only directly comparable when the same order of steps is followed for all analyses. Additionally, parameters such as circularity were not accounted for in these experiments. Operators can set minimum and maximum circularities to search for pores of specific shapes, however no conclusive evidence on the effect of pore circularity on cell proliferation was found during the execution of this thesis. ImageJ allows the option for outputting circularity data for all Analyze Particles runs, and so future iterations of this procedure may find interest in characterizing cell response as a function of both pore size and shape.

2.8 Conclusion

The aim of these studies was to improve and expand upon BVM lab scaffold characterization techniques. Specifically, experiments attempted to characterize the differences between the current manual method of measuring average PLGA scaffold fiber diameter and DiameterJ in an attempt to find a quicker, more automated replacement method that could reduce operator variability. Each method was used to measure a reference material of known dimensions to assess accuracy, to measure several

PLGA scaffold images fabricated in the BVM lab to assess the ability to perform in their intended application and to replicate past results for historical comparison purposes, and to measure several SEM images of electrospun fibers to compare each method to those used in other labs to assess how accurate direct comparisons between results might be. These results were presented as average fiber diameter values with accompanying standard deviation values, however this did not fully capture the true distribution of scaffold feature size. Reporting histograms of all measured fibers and pores can present information about size distribution within an image and scaffold, however for the sake of consistency with previous lab results and brevity, that was not included here.

DiameterJ did not prove to be any more accurate or quick in measuring fiber diameter compared to the current manual method. Issues with incomplete or inaccurate segmentation yielded results that were no more accurate than the manual method, and instances of necessary operator intervention slowed the process and introduced sections prone to variability via operator discretion. For this reason, **it is recommended that the BVM lab continue to use the manual fiber diameter measuring method for the sake of consistency and familiarity** until another method is discovered that is conclusively more appropriate for the needs of the lab. An updated fiber diameter measurement protocol is included in Appendix A compared to that used by Toni Pipes, and includes the exclusive use of a 4x4 circle template, specific contrast enhancement instructions, and succinct directions for making fiber selections within the circle template.

The goal of the pore area measurement experiments was to establish and test multiple protocols to assess the average pore area of the luminal surface of electrospun PLGA scaffolds produced in the BVM lab. This scaffold attribute has not been measured consistently in the lab, however tissue engineering literature suggests that pore area has a noticeable impact on cell adhesion and proliferation^{35,36,38–40,51}. A manual method using native ImageJ tools and a more automated method using the ImageJ plugin DiameterJ were tested on several SEM images of PLGA scaffolds as well as other fibrous materials to characterize the performance of each method.

Through measurement of a reference mesh of known dimensions and a representative PLGA scaffold, a minimum detectable pore area value of 19.22 pixels² and a contrast level of 15% through ImageJ's Analyze Particles and Enhance Contrast tools was selected as protocol standards. It was determined that the manual and DiameterJ methods produced significantly different average pore area for the majority of images measured, and that both experienced significant differences at different magnifications of the same image. Furthermore, both methods differed significantly from most published mean pore area values for images published in other works. Still, it is possible that either method could be used in the BVM lab; not as an absolute measure of pore area that could be accurately compared with literature values, but as a measure of relative comparison within the lab to characterize scaffolds and correlate with trends in cell response. Based on the work in this aim, it is recommended that the manual pore area method be used for all future pore area measurements in the BVM lab. While the protocol described herein (and included in Appendix C) does not currently produce results of satisfactory accuracy

or comparability to given values, it can be altered easily to accommodate future improvements.

3. FIBER DIAMETER AND PORE AREA REDUCTION STRATEGIES

3.1 Introduction

As stated previously, fiber diameter and pore size partially dictate cell adhesion and growth on a porous polymeric scaffold. Several sources suggest that fiber diameters at or below 1 μm are ideal for HUVECs, fibroblasts, and SMCs in terms of initial coverage on a scaffold^{31–34}. Additionally, there is evidence that the highest cell coverage for SMCs and 3T3s on porous scaffolds occurs at pore diameters within a ~50-100 μm range. Unfortunately, the current mean fiber diameter and pore size of PLGA scaffolds in the BVM has ranged between 4 and 10 μm , and is larger than values most research points to as being “ideal”, corroborated by BVM lab results. As such, the purpose of Aim 2 of this thesis was to investigate some techniques to lower the fiber diameter and pore size of PLGA scaffolds produced in the BVM lab.

3.1.1 Previous BVM Lab Research

Several researchers who have worked in the BVM lab have characterized the effect of various electrospinning parameters on scaffold fiber diameter and have tested various protocols with the intention of reducing fiber diameter as much as possible. Most of these attempts were concerned with altering processing parameters, such as gap distance, voltage, and flow rate. The electrospinning process was first established in the BVM lab by Colby James in 2009 and was initially characterized using 90:10 Poly(L-

lactic-co-caprolactone) (PLLA-CL). Wall thickness, Young's modulus, and average fiber diameter were all assessed, with the latter yielding values between 6 and 9 μm ⁷.

Tiffany Peña performed a rigorous materials selection process and determined that the lab would be best served by moving forward with 75:25 PLGA as the polymer of choice for electrospinning. These scaffolds were validated by their culturing for up to 6 days with HUVECs followed by analysis that showed cell coverage on the entire length of luminal surface as well as some penetration past the lumen. The optimal protocol used in this thesis defined voltage, flow rate, and polymer concentration parameters still in use today and was the basis for the current PLGA electrospinning protocol⁷⁰.

Yvette Castillo continued the work of reducing average PLGA scaffold fiber diameter by characterizing the interaction between several parameters and their combined effect on the scaffold. Based on these results a regression model was constructed that demonstrates a decrease in fiber diameter with decreasing flow rate, decreased applied voltage, and increased gap distance, in order of predictor strength. The model also predicted average fiber diameter values of 2.59 to 2.89 μm at optimal conditions⁷¹.

During this time the electrospinning setup was upgraded with the introduction of a new syringe pump, pump stand, and high voltage power supply with the added functionality of a negative polarity switch, detailed in the thesis of Deven Patel. The

optimal electrospinning protocol used parameters similar to those described by Tiffany, however the scaffolds produced showed statistically significant differences in fiber diameter and to a lesser extent porosity across a given set of scaffolds. It was also noted that while this difference was observed, the clear majority of fibers were between 2 and 3 μm , and the average pore area was $71 \pm 52 \mu\text{m}^2$ ¹¹⁴.

The most recent published research focusing on the BVM lab electrospinning setup was conducted by Toni Pipes, and was focused on characterizing the reproducibility of PLGA electrospun scaffolds using fiber diameter and compliance measurements as the metrics of interest. The protocol standard to the BVM lab at that point was reaffirmed for producing the smallest, most consistent fibers, and was the protocol used from that point until the present. With this protocol, an experimental study involving several replicates was performed by Toni to determine scaffold consistency. Average fiber diameter was observed to be $2.22 \pm 0.63 \mu\text{m}$, the smallest recorded across a large sample size in the BVM lab to date⁶.

Unfortunately, since the publishing of the Pipes electrospinning thesis, the average fiber diameter of scaffolds has steadily risen over time. Because applied voltage, flow rate, and gap distance have been tested extensively on the BVM lab electrospinning setup already, the experiments in this aim focused on altering other aspects of the scaffold fabrication process and were influenced by techniques to lower fiber diameter and pore size found in literature.

3.1.2 Current Research Focus

The electrospinning parameters investigated for PLGA scaffold optimization were related to the environment in which the electrospinning occurs, such as relative humidity and ambient temperature, and parameters effecting the composition of the polymer mixture itself, such as concentration of PLGA and solvent type. Environmental parameters were chosen because it is known that they have a significant impact on many types of polymer processing including electrospinning, and while humidity and temperature are recorded prior to each electrospinning session in the BVM lab, there have been few conclusions drawn or trends investigated^{100,101,103,124,125}. Solution parameters were chosen because they currently have not been investigated in the BVM lab in any capacity and previously published electrospinning literature suggests that solution properties are among the most influential parameters in the electrospinning process^{100,101,103}.

3.1.2.1 Environmental Parameters

Environmental conditions like relative humidity, ambient temperature, and ambient pressure are significant factors in any polymer processing technique. Many polymers are hygroscopic and attract water from the surrounding environment through either absorption or adsorption^{124,125}. This has implications for electrospinning as moisture content of a polymer is closely related to electrical properties, mechanical properties, and changes in morphology and composition due to possible hydrolysis^{73,125}.

Specifically, Pelipenko et al. observed a steady decrease in fiber diameter from 667 ± 83 to 161 ± 42 nm for a PVA-water solution and from 252 ± 39 to 75 ± 54 nm for a PEO-acetic acid mixture when relative humidity was increased from 4 ± 1 to $60\pm 1\%$ in 10% increments. In this case, higher humidity conditions lowered solvent evaporation rates and allowed the polymer jet to remain less viscous for a longer period of time, increasing the amount of time the jet can be elongated by voltage-induced stretching¹⁰³. De Vrieze et al. found similar decreases in fiber diameter with increases in relative humidity with poly(vinylpyrrolidone) (PVP) dissolved in ethanol for similar reasons. However, they also observed large amorphous mats of solidified PVP on samples at the highest humidity values tested due to low viscosity and overall fluid nature of the jet after it contacted the collecting surface¹⁰¹. While general accepted trends regarding the effect of humidity on electrospinning exist, there are differences in solution properties for different polymer/solvent combinations that must be considered.

De Vrieze also found an interesting relationship between temperature and fiber diameter: noticeably smaller fibers were formed at 283 and 303 K compared to those formed at 293 K. This was attributed to the two main electrospinning effects that are influenced by temperature, solvent evaporation rate and polymer chain rigidity. Evaporation rate decreases exponentially, and thus dominates in the 283 K electrospun product by allowing for longer voltage-induced elongation times. Additionally, polymer chain mobility increases at higher temperatures, decreasing viscosity, and thus the formation of a fibrous structure by elongation can occur more readily at 303 K^{60,101}. Furthermore, Yang et al. acknowledged these effects on electrospinning fiber diameter

with increases in temperature and observed similar results: Fibers produced from a mixture of PVP and ethanol were found to have average diameters that decreased from 830 ± 90 to 420 ± 30 nm between 20 and 40°C and then increased to 540 ± 40 nm after further increases in temperature to 50°C . Polyacrylonitrile (PAN) dissolved in dimethylacetamide (DMAc) was found to form fibers that decreased in diameter from 530 ± 80 to 280 ± 50 nm with incremental increases in temperature from 20 to 60°C ¹⁰⁰. This may be due to the temperature range selected for each polymer system, as there was no equivalent group to De Vrieze's 283 K experiment in the tests conducted by Yang. The temperature and humidity within the electrospinning chamber typically falls between 20 and 22°C and 40 to 60% RH, however these values are noticeably affected by the outside weather. As shown in the summaries above, even the fluctuations experienced in the BVM lab can produce noticeable changes in electrospinning results.

3.1.2.2 Solution Parameters

The process of electrospinning works by electrical conduction of the polymer solution across and physical space and relies on the predictable deformation of the resulting polymer jet; therefore, investigating the parameters that affect electrical and flow properties is quite important in exploring all options for scaffold optimization.

Because there is little data on the specific effect of solvent properties on PLGA for electrospinning, results from materials like PLLA were deemed acceptable for general comparison with previous BVM lab results. Maleki et al. investigated the effect of

solvent choice on electrospinning of poly(L-lactide) (PLLA) by mixing the polymer with either chloroform, dichloromethane, or 2,2,2-trifluoroethanol (TFE). It was found that fiber diameter was positively correlated with electrical conductivity and negatively correlated with density and surface tension of the base solvent to some degree, while there was no discernable fiber size correlation with vapor pressure or boiling point¹²⁶. Additionally, there are several sources that investigated mixtures of chloroform and other solvents like acetone and dichloromethane while using polymer systems similar to PLGA, such as PLLA that all achieve fiber diameters on the order of several hundred nanometers up to 2 μm ^{107,127–129}. These fiber diameter results are significantly smaller than what has been achieved in the BVM lab thus far, and so a subsection of these experiments was replicated to investigate their effect on PLGA. Combinations of chloroform and acetone were selected due to their availability on campus, relative safety of handling the base chemicals, and cost.

Several scaffolds were spun with changes to either electrospinning enclosure relative humidity levels or to the solvent content of the polymer solution to explore the effect each had on average fiber diameter and pore area. After spinning, scaffolds were sectioned and imaged using scanning electron microscopy and measured in ImageJ using techniques described herein. Based on previous electrospinning research with similar polymer systems, it was hypothesized that these scaffolds would exhibit significantly smaller fibers, which may have positive effects on the cell adhesion and proliferation of future BVM setups.

3.2 Methods

3.2.1 Solution Mixing and Electrospinning

Each spin was performed with a mixture of 75:25 PLGA (Sigma-Aldrich, P1941) at 15wt% unless noted otherwise. Approximately 0.7835 g PLGA was mixed in some combination of chloroform (Acros Organics, 326821000) and Acetone (VWR, BDH1101-4LP) as outlined in Appendix D. Standard PLGA-chloroform produces a solution of approximately 3 mL, however this amount differs with chloroform-acetone mixtures due to differences in density and thus differences in total solution volumes, resulting in variable total spinning times. After mixing for 24-48 hours on an orbital shaker table each solution was spun in accordance with the standard BVM lab protocol as outlined in Appendix E.

3.2.2 Environmental Parameters

Preliminary experimentation with humidity alterations within the electrospinning chamber and subsequent electrospinning was performed as a pilot study. This was done to investigate the effect of controlled relative humidity at various levels on scaffold morphology and to determine the feasibility of altering the humidity within the large chamber for each spin. Increasing humidity above ambient values was achieved with a terrarium humidifier/fogger (Impresa Products, 1CO9582Y) using distilled water. Attempts to lower humidity below ambient values was performed with a thermoelectric dehumidifier (Ivation, IVAGDM20), both controlled with a greenhouse humidity and

temperature controller (Inkbird, IHC-230) (Figure 49). The temperature and humidity setup will be further explored in Chapter 4, towards the aim of making the overall electrospinning protocol more consistent.

Figure 49. The BVM lab electrospinning setup with terrarium humidifier and controller in use.

Scaffolds were spun at the highest and lowest achievable relative humidity values of 79 and 38% to establish a general trend in PLGA fiber morphology and size, with all other electrospinning parameters held constant with standard electrospinning protocol values (Table XIV).

Table 14. Samples and Parameters for Environmental Parameters Trial

Spin Number	Material	Solvent	Concentration (wt%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)	Relative Humidity (%)
1E	PLGA	Chloroform	15	5.5	-12	10	38
2E	PLGA	Chloroform	15	5.5	-12	10	79

3.2.3 Solvent Combinations

Preliminary experimentation with electrospinning PLGA solvated in chloroform-acetone mixtures was performed with 100% acetone and 1:1 and 2:1 chloroform-acetone mixtures by volume; this was compared to standard 100% chloroform scaffolds and used density values of 0.791 and 1.49 g/mL for acetone and chloroform, respectively (Table XV).

Table 15. Solvent ratios to achieve 15wt% PLGA electrospinning solution

Solvent(s)	Ratio	Vol. CHCl ₃ (mL)	Vol. Acetone (mL)	Total Volume (mL)
Acetone	-	0.00	5.63	5.63
Chloroform:Acetone	1:1	1.95	1.95	3.90
Chloroform:Acetone	2:1	2.35	1.18	3.53
Chloroform	-	3.00	0.00	3.00

The purpose of these preliminary scaffolds was to assess the feasibility of these solvent combinations and to determine whether future optimization of a protocol regarding their use was warranted. All solvent ratios were measured by mass using standard bulk density values and using predetermined volumes of each solvent. The details of each spin were documented and all parameters not directly related to the polymer solution were held constant (Table XVI).

Table 16. Samples and Parameters for Solvent Combinations Trial

Spin Number	Material	Solvent	Concentration (wt%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
1S	PLGA	Acetone	15	5.5	-12	10
2S	PLGA	Chloroform:Acetone, 1:1	15	5.5	-12	10
3S	PLGA	Chloroform:Acetone, 1:1	17	5.5	-12	10
4S	PLGA	Chloroform:Acetone, 2:1	15	5.5	-12	10

While acetone was originally chosen because of its previous use with PLLA, accessibility, and relative safety, it was later discovered that mixtures of chloroform and acetone will undergo a highly exothermic condensation reaction in the presence of a basic environment, commonly when in contact with potassium hydroxide or calcium hydroxide¹³⁰. A handling and disposal protocol for chloroform:acetone combinations was developed through collaboration with the Cal Poly Environmental Health and Safety Department to preserve the safety of all those involved, and is included in this thesis (Appendix F).

3.3 Results

3.3.1 Environmental Parameters

Upon testing the humidifying and dehumidifying systems inside the electrospinning enclosure, it was determined that the minimum and maximum relative humidity values that could be accurately achieved were 38 and 79%, respectively. These parameters were used to test the initial effects of humidity on the scaffold fabrication

process and the resulting fiber diameter and pore size. Details and observations of each spin during and after the electrospinning process were documented and are presented along with quantitative measurements of average fiber diameter and pore area.

Spin 1E

Purpose:

The parameters used in Spin 1E were identical to those in the standard electrospinning protocol (Appendix E), and were spun at the lowest achievable relative humidity of 38% through use of the dehumidifier. This test was done to establish a fiber diameter (Appendix A) and pore area (Appendix C) standard for the lower bound of relative humidity achievable in the lab, and to compare with the high humidity Spin 2E to observe any effect of RH on fiber diameter and pore size.

Parameters:

Material	Solvent	Concentration (wt%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)	Relative Humidity (%)
PLGA	Chloroform	15	5.5	-12	10	38

Observations:

All aspects of the electrospinning process were observed to be very similar to that of a standard PLGA spin, likely because 38% RH is much closer to typical ambient conditions than the artificially heightened humidity of Spin 2E. SEM images show a fairly standard fibrous, porous morphology, and average fiber diameter and pore size measurements were recorded (Figure 50, Table XVII).

Figure 50. SEM images of Spin 1E at 60x (left) and 600x (right) at proximal (top), middle (middle), and distal (bottom) positions along the scaffold.

Table 17. Fiber Diameter and Pore Area of Environmental Parameters Study, Spin 1E

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	6.45	3.58	639.88	1748.71
Middle	5.92	2.95	286.63	360.70
Distal	8.48	2.78	758.23	1243.86
Average	7.13	3.27	440.95	998.62

Spin 2E

Purpose:

Spin 2E was electrospun at 79% RH as a direct, high humidity comparison to Spin 1E regarding fiber diameter and pore area as well as general electrospinning process observations. 79% RH was selected due to it being the highest humidity reliably achieved by the terrarium fogger in the electrospinning chamber. Previous electrospinning literature has shown that increased humidity has led to decreased fiber diameter in multiple electrospun materials^{101,103}.

Parameters:

Material	Solvent	Concentration (wt%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)	Relative Humidity (%)
PLGA	Chloroform	15	5.5	-12	10	79

Observations:

Due to the method of humidification provided by the terrarium fogger/humidifier, the electrospinning chamber was partially filled with a visible layer of fog when the humidity had reached the desired percentage. During electrospinning, it was observed

that the droplet that remains resting on the beveled tip of the needle was initially much smaller than those usually observed at ambient relative humidity, however it gradually increased in size to near-normal over time. To preserve the elevated relative humidity the humidifier was turned on automatically by the greenhouse environment controller, and during these times the bead reduced in size until the humidifier was switched off, at which point the bead would immediately grow.

Removing the scaffold from the mandrel was much more difficult than PLGA spins done at ambient conditions to the point that only small portions of the scaffold could be cut from the mandrel for sectioning and microscopy while large sections of intact scaffold were impossible to remove. After weeks of desiccation the scaffolds had become no easier to remove, and upon cutting the scaffolds apart to free the mandrel for future use it was found that several patches of scaffold had pulled away from the larger pieces and were stuck to the mandrel firmly, only releasing with washes of isopropyl alcohol (Figure 51). This is a factor that makes higher relative humidity values unattractive from a practical standpoint despite the potential for smaller average fiber diameter. SEM images showed this pull-out of PLGA as well as a relatively normal fibrous structure with some unexpected alignment in the direction of rotation (Figure 52, Table XVIII).

Figure 51. Mandrel from Spin 1E after scaffold removal showing adhered patches of fibrous PLGA.

Figure 52. SEM images of Spin 2E at 60x (left) and 600x (right) at middle (top), and distal (middle) positions along the scaffold, as well as images at 50x (bottom left) and 60x (bottom right) that show disturbances where patches of scaffold tore out and remained on the mandrel.

Table 18. Fiber Diameter and Pore Area of Environmental Parameters Study, Spin 2E

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Middle	4.31	1.53	327.27	452.93
Distal	8.04	2.44	513.41	710.38
Average	6.70	2.80	390.10	555.84

Fiber diameter and pore area results were compared directly between scaffold averages using a general linear model with Tukey pairwise comparisons. It was determined that the average fiber diameter of 1E and 2E did not significantly differ, however the average pore area results did (Figure 53 and 54). This suggested that humidity does not affect fiber size for PLGA dissolved in chloroform at the parameters specified in the standard BVM protocol.

Figure 53. Box and whisker plot of humidity trial fiber diameter results. Average fiber diameter did not differ significantly between 1E and 2E.

Figure 54. Box and whisker plot of humidity trial pore area results. *, $p < 0.05$ between groups.

Next, several PLGA scaffolds were spun with varying solvent concentrations to investigate their impact on fiber diameter, pore area, and general scaffold morphology.

3.3.2 Solvent Combinations

After approval of the chloroform:acetone mixing procedure from the Cal Poly Environmental Health and Safety Department, the solutions described in Table XVI were mixed and spun to observe the effect of solvent properties on electrospinning results. Details and observations of each spin during and after the electrospinning process were documented and are presented along with quantitative measurements of average fiber diameter (Appendix A) and pore area (Appendix C) using the manual characterization methods described in Chapter 2.

Spin 1S

Purpose:

Based on the research of Zeng et al. the decision was made to electrospun PLGA with alternative solvent compositions as a method by which to decrease average fiber diameter¹²⁷. While Zeng et al. solely used a 2:1 chloroform:acetone ratio, chloroform had been the only solvent used in PLGA electrospinning in the BVM lab and thus pure acetone and a 1:1 solvent ratio were tested as well; Spin 1S was the pure acetone scaffold, and acted as a direct comparison between the properties of acetone and chloroform and an assessment of their respective effects on scaffold fabrication and morphology.

Parameters:

Material	Solvent	Concentration (wt%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PLGA	Acetone	15	5.5	-12	10

Observations:

Full polymer coverage on the mandrel was achieved at standard BVM electrospinning conditions initially, however the voltage was incrementally increased to -15kV to reduce dripping from the needle tip as the spin progressed. Dripping did not fully cease at -15kV and so the flow rate was also decreased to 4.5 mL/hr. The dripping observed in Spin 1S was of much finer, more frequent droplets compared to larger and more gradual, elongated drips observed in most PLGA spins mixed with just CHCl₃. In

addition to an excess of polymer solution at the tip causing dripping, it was observed that a droplet of solution would begin to solidify while resting on the beveled needle tip, and a more fluid droplet would form on its underside, preventing it from being pulled into a jet and instead causing more excessive dripping. The solidifying droplet would either be pulled away from the needle tip at some point or it was manually cleared after switching the syringe pump and voltage supply off temporarily.

SEM images yielded a much different fiber morphology than that of typical PLGA fibers formed from a pure CHCl_3 solution. The acetone solution yielded much smaller fibers that ranged between approximately 500 and 1100 nanometers with beads several times larger than the fibers, on the order of 1 to 3 μm . Fiber diameter and pore area measurements were made at 2000x to yield a more accurate measurement due to small feature size at 600x, and are comprised of fiber and bead measurements (Figure 55, Table XIX).

Figure 55. SEM images of Spin 1S at 100x (top left), 600x (top right), 1000x (bottom left) and 2000x (bottom right) all from the proximal position of the scaffold.

Table 19. Fiber Diameter and Pore Area of the Solvent Combinations Study, Spin 1S

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	1.34	0.79	50.73	42.43

Spin 2S

Purpose:

Spin 2S was mixed with a 1:1 ratio of chloroform and acetone as an intermediate value between pure acetone and the 2:1 ratio described in the work of Zeng et al¹²⁸.

Solvent mixtures of various ratios are common in electrospinning literature, and are often done to combine desirable characteristics of both solvents used^{37,107,128}. In this way, Spin 2S represented an attempt to combine any desirable outcomes of Spin 1S with the standard 100% chloroform PLGA scaffold results.

Parameters:

Material	Solvent	Concentration (wt%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PLGA	Chloroform:Acetone, 1:1	15	5.5	-12	10

Observations:

The electrospinning behavior of Spin 2S was similar to that of Spin 1S regarding dripping and the necessary changes to applied voltage (increased to -14kV), and in the dripping characteristics including the formation of a partially solidified droplet that prevented a jet from forming. SEM images showed that the fiber morphology of Spin 2S was a combination of Spin 1S and standard PLGA scaffolds (Figure 56). Fibers easily visible at 600x were densely packed together on the luminal surface of the scaffold, and had some evidence of wider sections that appeared similar to the beads of Spin 1S (Figure 55 and 56, Table XX).

Figure 56. SEM images of Spin 2S at 100x (left) and 600x (right) at the proximal position along the scaffold.

Table 20. Fiber Diameter and Pore Area of the Solvent Combinations Study, Spin 2S

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	2.17	1.05	210.33	371.33

Spin 3S

Purpose:

Spin 3S was mixed and spun after the results of Spin 1S and 2S had been obtained; The beaded fibers observed in the previous spins were assumed to be caused by a decrease in solution viscosity relative to surface tension; viscosity was increased in Spin 3S by increasing the polymer concentration from 15 to 17wt% while keeping all other solution parameters constant. Polymer mass was held constant at 0.7835 g as specified in the electrospinning protocol, and solvents were adjusted accordingly: the volume of both acetone and chloroform decreased from 1.95 mL to 1.68 mL each.

It is accepted that many polymer-solvent combinations experience a similar trend in electrospinning product morphology; specifically, as polymer concentration increases from 0% the structure produced forms beads, beaded fibers, and then smooth fibers^{96,111}. The relatively low frequency and size of beads in Spin 2S suggested that 15wt% in a 1:1 CHCl₃:acetone solution was near the boundary between beaded and smooth fiber regimes.

Parameters:

Material	Solvent	Concentration (wt%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PLGA	Chloroform:Acetone, 1:1	17	5.5	-12	10

Observations:

Similar to previous descriptions, electrospinning for Spin 3S required increases in applied voltage up to -14kV to reduce dripping and maintain a constant polymer jet. SEM images suggested that increasing the polymer concentration from 15 to 17wt% did in fact eliminate the presence of beads on fibers however the significant increase in average fiber diameter from 2.17 to 3.81 μm was not desirable (Figure 57, Table XXI).

Figure 57. SEM images of Spin 3S at 60x (left) and 600x (right) at proximal (top) and distal (bottom) positions along the scaffold.

Table 21. Fiber Diameter and Pore Area of the Solvent Combinations Study, Spin 3S

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	3.59	1.06	64.95	55.20
Distal	4.03	0.91	77.23	112.96
Average	3.81	1.00	70.44	85.94

Spin 4S

Purpose:

Spin 4S was performed as the final CHCl₃:acetone combination which was the same 2:1 ratio used by Zeng et al. with PLLA to form fibers with diameters well below the results achieved in the BVM lab thus far. Like the increased viscosity achieved through increased polymer concentration in Spin 3S, the relative viscosity to surface tension was increased with an increased chloroform concentration. In this way, Spin 4S was expected to also produce less beads than Spins 1S and 2S, and continue any observable trends in fiber diameter and morphology regarding solvent content with spins 1S, 2S and 3S, and standard 100% chloroform PLGA spins.

Parameters:

Material	Solvent	Concentration (wt%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PLGA	Chloroform:Acetone, 2:1	15	5.5	-12	10

Observations:

During electrospinning the voltage was increased to -13.5kV to reducing dripping, and it was observed that the droplets that formed were more elongated and generally more similar to those formed with solutions of purely PLGA and CHCl₃ compared to the other trials performed with acetone and CHCl₃:acetone mixtures.

SEM images revealed some beaded fibers although the ratio between the bead and fiber size was not as high as in previous spins. The density and presence of smaller fibers decreased compared to Spin 1S and 2S, and the average fiber diameter was much closer to that of Spin 3S (Figure 58, Table XXII).

Figure 58. SEM images of Spin 4S at 60x (left) and 600x (right) at proximal (top) and distal (bottom) positions along the scaffold.

Table 22. Fiber Diameter and Pore Area of the Solvent Combinations Study, Spin 4S

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	2.99	1.95	195.59	241.15
Distal	4.21	2.57	207.37	255.24
Average	3.59	2.32	199.18	244.64

Fiber diameter and pore area results were compared directly using a general linear model with Tukey pairwise comparisons: It was determined that the fiber diameter values of 1S and 2S differed significantly from those of 3S and 4S, and that the pore area values of 1S and 3S differed significantly from those of 2S and 4S as well (Figure 59 and 60).

Figure 59. Box and whisker diagram of solvent trial fiber diameter. \$, $p < 0.001$ between 3S. #, $p < 0.05$ between 4S. @, $p < 0.001$ between 4S.

Figure 60. Box and whisker diagram of solvent trials pore area. \$, $p < 0.001$ between 4S. #, $p < 0.05$ between 2S. @, $p < 0.01$ between 2S.

3.4 Discussion

3.4.1 Humidity

The results of the humidity test showed that fiber diameter did not differ significantly between scaffolds spun at 38 and 79% relative humidity, while the measurements of pore area did. The effect of humidity on fiber diameter and general morphology is complex and may have been dictated by several factors: Chloroform and PLGA are both considered hygroscopic substances, and the presence of jets and beads of solution in the ambient environment of the high humidity conditions of Spin 2E may have provided an opportunity for water to be absorbed by the solution¹³¹. This can lead to an increase in fiber diameter due to incomplete drying and thus incomplete thinning prior to contact with the mandrel. Similarly, the absorption of water may have changed the viscosity or conduction of the solution as water has a much high dielectric constant than

chloroform (80.0 compared to 4.8), further altering fiber formation during electrospinning. However, there was no significant difference in fiber diameter between the low and high humidity groups. The difference in pore area may simply be due to the previously described inaccuracies of the current pore area measurement method, and so these results were considered secondary to those of the fiber diameter measurements.

It is possible that the effect of humidity and water absorption differs in intensity based on polymer and solvent combination, and that PLGA in chloroform may be particularly resistant to its effects. It is possible that a PLGA-chloroform solution would not change significantly above a particular relative humidity value, and that both 38 and 79% RH tested in this experiment were above that value. This speculation is corroborated by data presented for electrospun PEG, PCL, and poly(carbonate urethane) (PCU): It was determined that each polymer had a broad range of relative humidity in which relatively stable fibers formed, and that any conditions outside of this range lead to beaded, broken, or a distinct lack of fibers deposited. The ranges of acceptable relative humidity values in this work include 50% to greater than 75%, while the range of TH for PCU before a lack of fiber formation existed from 20% to 50%¹⁰². This suggested that a range of relative humidity values in which an electrospun polymer is largely unchanged in performance was not only possible, but subject to change based on the polymer. Unfortunately, the humidity range tested was the extent of the BVM lab humidity-altering capabilities and all other methods attempted to further lower humidity were not successful.

In summary, there existed no significant difference between fiber diameter results from the low and high humidity electrospinning environments. While the exact reason is unknown, it appears that the only noticeable difference between the two scaffolds was the relative difficulty of their removal from their respective mandrels. For this reason, it is advised that the BVM lab attempts to spin at low RH values to mitigate instances of humidity-induced scaffold removal issues.

3.4.2 Solvent

In contrast to the humidity electrospinning trials, the solvent composition trials did yield scaffolds of significantly different fiber diameters and pore areas. It was found that 15wt% PLGA in either 100% acetone or a 1:1 chloroform acetone ratio yielded densely packed beaded fibers which appeared to be among the smallest fibers recorded in the BVM lab alongside the results of Toni Pipes (2.22 μm)⁶. Scaffolds spun at 17wt% PLGA in a 1:1 ratio or 15wt% in a 2:1 ratio yielded larger, smoother fibers that more closely resemble those of standard BVM lab PLGA scaffolds.

These differences in fiber diameter and morphology could be explained by a few underlying effects: Firstly, acetone has higher electrical conductivity and lower surface tension and viscosity than chloroform, two key solution parameters in electrospinning¹⁰⁷. Acetone also has a slightly lower boiling point, which is often used as an approximation for relative volatility where a lower boiling point indicates a higher volatility¹⁰⁷. Simply put, the stretching and elongating of a polymer jet during electrospinning is caused by the

electrostatic forces imparted by the electrospinning setup by pulling the solution (and the buildup of charges contained within it) towards the grounded surface. Coulomb's law states that the strength of an electric field increases exponentially as the distance between two charges decreases, and so the portion of the jet is drawn towards the grounded surface with greater force, stretching it away from the jet portion further from the grounded surface, all while the entire jet is moving towards said surface¹³². The stretching of a jet is contested by the forces of surface tension and viscosity of the solution, which resist both the formation of additional surface area and the general shearing of the liquid polymer solution^{96,102,110}. As such, the previously stated properties of acetone suggested that solutions mixed with acetone rather than chloroform were more readily able to overcome the forces of surface tension and viscosity and thus elongate more during the travel distance of a polymer jet, resulting in smaller fibers. As noted previously, acetone also has a slightly lower boiling point than chloroform (56 vs 61 °C), a property that is commonly associated with volatility and evaporation rate¹⁰⁷. As the polymer jet moved towards the collector the solvent evaporated, solidifying the fibers and preventing any further stretching or elongating.

The results of the solvent composition experiment described in this thesis showed that increasing acetone content led to the formation of smaller fibers on average, as well as increased the instance of bead formation. This suggests that the effect of lower surface tension and viscosity of acetone on decreasing fiber size was more impactful than any possible premature fiber solidification due to the increased volatility of acetone compared

to chloroform. However, this phenomenon of premature solidification should be considered if future solvent comparisons are performed.

The formation of beads during electrospinning is also caused by the interplay between forces acting on the polymer, specifically between surface tension and viscoelastic forces. A jet of polymer solution moving towards a grounded surface can be modeled as a column of liquid, and as such is subject to the Plateau-Rayleigh instability which describes the breakup of the stream and formation of beads in the same way that droplets form at the end of a stream of pouring water (Figure 61)^{88,90-92}.

Figure 61. A (a) simulated and (b) real-world experimental example of the Plateau-Rayleigh instability occurring over time in a polymer suspended between 2 parallel plates¹³³.

This phenomenon is caused by the forces of surface tension attempting to minimize surface energy and form spherical volumes of fluid. This is subsequently

resisted by viscoelastic forces which oppose the rapid movement of shearing fluid. While acetone has lower surface tension and viscosity than chloroform, the difference in viscosity is much greater. Casasola et al. reported that the viscosity of chloroform is nearly twice that of acetone, 0.563 compared to 0.308 mPa*s, while their respective surface tension values are 27.2 and 23.3 mN/m¹⁰⁷. This suggested that the relative difference in surface tension and viscous forces in acetone was such that the minimization of surface energy in the polymer jet overcame the resistance to liquid movement, Rayleigh instability occurred, and beads formed along relatively small fibers. This is corroborated by the fiber morphology presented in Figures 55 and 56, and the assertion that decreasing the surface tension/viscosity ratio through the addition of more chloroform results in less beading is supported by Figure 58, which shows smooth, unbeaded fibers. This is further supported by electrospinning literature results that show a marked decrease in fiber diameter with the addition of a variety of surfactants, added with the intention of decreasing solution surface tension^{110,111,134}. Similarly, additives such as salts and other charged particles have been shown to decrease fiber diameter by increasing the number of charge carriers in solution, and thus electrostatic force exhibited during electrospinning^{111,128,135}.

The role of viscoelastic forces in the formation of smooth fibers also explained why increasing the polymer content of a given solution composition from 15 to 17wt% in 1:1 chloroform:acetone led to smaller, less frequent beading along the fibers of the scaffold, as the increase in polymer concentration led to a direct increase in viscosity. While attempts made to quantify viscosity of each solution were ultimately unsuccessful,

a noticeable increase in apparent viscosity was observed with increasing chloroform content when handling each solution in preparation for electrospinning. The results from the solvent study showed that decreasing surface tension and viscosity of a solution through alterations in solvent composition results in a noticeable decrease in fiber diameter and pore size for electrospun PLGA.

3.5 Conclusion

This chapter discussed the aim of reducing scaffold fiber diameter and pore area by investigating humidity and solvent composition electrospinning parameters. Previous BVM lab work on the subject had pertained to the parameters of the electrospinning setup itself and established optimized settings for these parameters, however the effect of environmental and solution parameters had not been explored to nearly the same degree. This chapter serves as a summary of preliminary experimentation regarding the effect of relative humidity and solvent composition on the effect of PLGA scaffold morphology, fiber diameter, and pore size.

The trials concerning the effect of relative humidity of the electrospinning enclosure on PLGA scaffold results suggested that **no significant difference existed between RH values of 38% and 79%.** This finding is contrary to several literature sources that cite several possible effects of increased moisture content, resulting in increases and decreases in fiber diameter based on polymer and solvent system. Additionally, there existed several consistency issues with the general electrospinning

setup and environmental control of the enclosure specifically during these experiments that will be described in the following chapter; These circumstances may have obscured any effect that humidity may have had, and future experimentation with both humidity and temperature is recommended when the consistency of the system can be controlled more precisely. These recommendations will be further discussed in Chapter 7.

The trials regarding the effect of solvent composition were much more conclusive, and suggest a clear trend in scaffold results with solvent properties. The addition of acetone caused a decrease in solution viscosity noted qualitatively during electrospinning and that was assumed to have caused the resulting difference in fiber diameter observed. Scaffolds spun with PLGA dissolved in pure acetone resulted in fibers noticeably smaller than those ever spun in the BVM lab before, however these fibers were heavily laden with large beads. Combining acetone and chloroform resulted in a reduction in beading and increase in fiber diameter compared to a pure PLGA-acetone solution, and more densely-packed fibers than those normally observed with PLGA dissolved in pure chloroform. These results suggest that **PLGA scaffolds spun with a 1:1 chloroform:acetone ratio of polymer concentration between 15 and 17wt% should be pursued** to determine the effect of morphological scaffold differences with standard PLGA scaffolds on cell response during a full BVM setup. Additionally, chloroform and acetone are not the only solvents that dissolve PLGA and are certainly not the only solvents found to have been mixed in electrospinning literature^{107,128}. Through an understanding of the effects of viscosity, surface tension, solvent evaporation rate, and many other solution properties it is recommended that future research into other

solvents and solvent combinations be attempted to properly characterize the effect each has on PLGA specifically. For the purposes of this thesis, and specifically Aim 2, the reduction of fiber diameter and pore area through the alteration of relative humidity was ineffective, however experimentations with varying solvent compositions were successful. Because of this, it is recommended that the BVM lab move forward with two types of testing: 1). Use PLGA scaffolds spun with various ratios of acetone and chloroform to investigate the impact of reduced fiber diameter and pore area on BVM performance, and 2) Investigate the effect of other solvents and solvent combinations on electrospun PLGA scaffolds.

4. INVESTIGATING INCONSISTENCIES WITH THE CURRENT ELECTROSPINNING PROTOCOL

4.1 Introduction

The BVM lab electrospinning protocol has been in place in some form since the process' inception in the lab in 2009⁷. The current protocol followed by all electrospinning lab members has been modified multiple times and generally provides very clear instructions that will guide one to successfully electrospin a serviceable polymer scaffold (Appendix D,E)⁶. There are a few aspects of the protocol, however, that were either extraneous or unclear, which led to varied interpretation based on user. This has indirectly created a set of informal guidelines passed down by word of mouth that may have been distorted as time goes on and operators change.

Aim 3 of this thesis was to reduce process variability by developing a less ambiguous electrospinning protocol for the BVM lab. The purpose of this chapter was to identify and eliminate any instances of uncertainty or variability both within the written BVM electrospinning protocol and in the practical use of the electrospinning system, and to remove any extraneous, confusing, or otherwise unnecessary information to create a revised protocol for all future electrospinning use. In this way, the variability between spins and between operators will be reduced. The following introduction will present several aspects of the electrospinning setup, including mixing PLGA solution, the position of sensitive electrical components, attempts to control environmental conditions

within the electrospinning chamber, and scaffolds removal after electrospinning, and potential issues associated with them. The proposed solutions for these issues will be covered in detail in later sections within this chapter.

4.1.1 Solution Mixing

Polymer concentration in an electrospinning solution is an important parameter for obtaining scaffolds of proper size and morphology; concentration is one factor which dictates solution viscosity, responsible for resisting polymer jet deformation and the formation of beads^{96,102}. In the pursuit of consistent polymer concentration measurements, the BVM electrospinning protocol had directed operators to abide by an unpractically high degree of PLGA-weighing precision that noticeably lengthened the time necessary to complete the solution mixing process (Appendix D). Specifically, the protocol called for users to weigh PLGA to an accuracy of ± 0.0001 g, or 100 μm . PLGA pellets weigh approximately 10 times this, and so operators were spending 30 minutes or more switching out pellets one at a time from their weigh boat until the described level of precision was achieved. Additionally, while the protocol stated the proper amounts of PLGA and chloroform necessary to mix a solution to spin a standard scaffold at fixed concentration parameters, there was no mention of a general formula for determining proper constituent amounts if other scaffold properties were desired. Both issues were rectified in the execution of the experiments described in this chapter.

4.1.2 Experimental Setup

It was observed that polymer coverage was significantly lesser on the end of the mandrel furthest from the operator (referred to as the “distal” end) compared to the “proximal” end, resulting in scaffolds of uneven wall thickness (Figure 62). Scaffolds in the BVM lab are spun with a specific amount of PLGA to ensure they are strong enough to resist the stresses of the BVM setup, and scaffold sections of unexpectedly lower wall thickness have led to tearing and failure of BVMs in the past.

It was speculated this wall thickness discrepancy was due to some errant electrostatic force acting on the jet during the electrospinning process: The driving force behind electrospinning is the electrostatic attraction between the charged polymer mixture and grounded conductive mandrel surface, and as such any interference of this field can impact the path and result of the elongating fiber jet during the process. Several sources described attempts to use induced magnetic fields to alter the morphology of electrospun fibers by shaping the jet to alter fiber diameter, prevent jet instability, or to induce fiber alignment when deposited onto a surface^{136–138}. It was possible that the wire used to deliver a charge from the voltage source to the needle was not completely shielded, and thus a magnetic field formed from the current flowing through the wire emanated from the location of the wire. Prior to investigations this wire was located on a plane normal to the needle tip where the needle fit through a hole in the electrospinner housing. The clip attached to the needle was positioned directly down from the needle tip, however the wire made a “J” shape around the distal end of the electrospinning enclosure before connecting to the power supply (Figure 62).

Figure 62. Electrospinner orientation terms relative to the position of the electrospinning collector (left) and the position of the red charge-carrying wire during electrospinning (left).

4.1.3 Environmental Control

Another source of inconsistency within the BVM lab electrospinning setup was identified regarding the state of the environmental conditions inside the electrospinning enclosure (Figure 63).

Figure 63. Image of the current electrospinning setup.

The enclosure is not sealed in any way and thus the temperature and humidity is essentially that of the rest of the laboratory, which is significantly impacted by changes in weather, particularly the sharp increase in humidity experienced with rain. Previously recorded relative humidity values in the enclosure have ranged between 30 and 60%. As stated previously, electrospinning results in literature have been shown to be significantly affected by temperature and relative humidity, however this was not found to be the case in the experiments described in Chapter 3. However, for the sake of consistency and to aid in scaffold removal, a method was sought to provide consistent humidity and temperature control to electrospinning operators for more consistent results overall and for the added ability to tailor these environmental factors to achieve specific results^{96,101,103}.

4.1.4 Scaffold Removal

After electrospinning, scaffolds are placed in a desiccator for 24-48 hours until all traces of residual solvent have evaporated. The scaffold is then removed from the mandrel manually by sliding the scaffold down the length of the mandrel; ideally this process would occur in a gentle manner so as not to disrupt the size and shape of the scaffold or the fibers that compose it. However, another pervasive yet inconsistent issue with the electrospinning process was the seemingly random fluctuations in the amount of force required to remove a scaffold from the mandrel. The current protocol stated that operators are to sand the surface of each mandrel with 1200 grit sand paper to normalize surface roughness prior to electrospinning. This method was subject to variation based on operator handling, and improper cleaning after sanding has led to the appearance of

microscopic metallic particles on SEM images of scaffolds in the past. Quickly dipping the scaffold in ethanol rendered the scaffold easier to remove in the past, however ethanol increases the pliability of the fibers and may have changed their orientation when handled; because of concerns that the scaffolds are disrupted by this method, ethanol dipping has largely been avoided. Additional attempts to mitigate these fluctuations include several handling techniques based on “feel” and operator experience. However, using these techniques has only provided a slight improvement in ease of scaffold removal, and a more effective, consistent solution is required.

4.2 Methods and Results

Each step of the electrospinning process was investigated for inconsistencies and sources of variation, with a focus on the issues introduced above. These steps included mixing the polymer solution, preparations taken before electrospinning, the process itself, and removing the fabricated scaffold from the mandrel. They are presented in the order in which they are performed when executing the electrospinning protocol and all experiments, observations, and results are presented in full before another topic is introduced.

4.2.1 Solution Mixing

The current solution mixing protocol states that PLGA should be weighed out to 0.7835 grams to the nearest ± 0.0001 g, which when mixed with 3 mL of chloroform yields a solution with 15wt% PLGA. However, in practice, achieving such high PLGA

mass precision is both impractical and not necessary. PLGA is purchased in discrete pellets which weigh significantly more than 0.0001 g, meaning that the only way to achieve such a stringent mass requirement is to meticulously weigh, exchange pellets that appear to be of different sizes, and weigh again until the appropriate mass is achieved. This can take more than 30 minutes, which is unreasonable and exposes the hygroscopic PLGA to ambient humidity and increasing the risk of detrimental water contamination in the polymer solution. Additionally, the scale used for these measurements regularly fluctuated by ± 0.0001 g with no apparent stimulation, suggesting that measurements to such a degree aren't accurate anyway. Finally, the relatively inaccurate measurement of 3 mL of chloroform performed with a 10 mL syringe with 0.2 mL graduations obviated the supposed precision provided by the PLGA mass measuring requirements. Since this issue came to light several scaffolds have been spun using PLGA measured with no greater than ± 0.004 or an approximately 0.5% deviation from the target mass of 0.7835 g with acceptable results and no apparent inconsistencies. Additionally, the new protocol includes the general equations for determining solvent amounts for a given solution concentration (Eq. 2 and 3).

$$M_t = M_p + (V_1 * \rho_1) + \cdots (V_n * \rho_n) \quad (2)$$

In which M_t is the total mass of the solution, M_p is the polymer mass, V and ρ are the volume and density of a solvent, and n is the number of solvents. Assuming the polymer mass and concentration in solution as a wt.% are known target values, the total solution mass can be calculated and this equation can be used to determine the total

required volume of any number of solvents. Eq. 2 can be manipulated to form the following:

$$M_s = V_1 * \rho_1 + \cdots V_n * \rho_n \quad (3)$$

In which M_s is the combined mass of the solvents. Knowledge of all the solvent densities as well as the volume ratio between solvents allows one to reduce all volume terms into a single variable through substitution and solve. This equation could also be manipulated to determine a polymer mass from a given total solvent volume and polymer concentration value, and so on. An example is provided in Appendix I.

4.2.2 Experimental Setup

4.2.2.1 Eliminating Magnetic Interference

The discrepancy in wall thickness between proximal and distal ends of an electrospun scaffold was hypothesized to be caused by the uneven position of the wire carrying a charge to the needle tip with respect to the location of the needle (Figure 64, shown in red). This wire carries a substantial charge which creates a magnetic field around the wire, and its presence on the distal side of the electrospinning enclosure near the needle tip repels the polymer jet, leading to a larger buildup of PLGA on the proximal end of the mandrel. This was resolved by simply moving the wire outside of the container and positioning it in such a way that it ran parallel to the syringe and needle, eliminating any uneven electrospinning results due to wire proximity (Figure 64).

Figure 64. Comparison between the old (left) and new (right) positions of the red charged wire.

In addition to visual confirmation of a more aligned polymer jet, there was a significant difference in wall thickness measured across the length of the scaffold in which the thickness of the proximal end was $456.81\ \mu\text{m}$ compared to $309.62\ \mu\text{m}$ of the distal end. This contrasted with the results of the scaffold spun with the new wire placement, in which the proximal and distal end wall thicknesses were $244.15\ \mu\text{m}$ and $297.19\ \mu\text{m}$, respectively. These values were not found to be significantly different, while old wire placement scaffold wall thickness did (Figure 65 and 66).

Figure 65. SEM images and boxplots showing difference between proximal and distal scaffold wall thicknesses spun with the old wire placement. ***, $p < 0.001$ between groups.

Figure 66. SEM images and boxplot of scaffold wall thickness spun with the new wire position. There is no significant difference between scaffold locations at the new position.

Next, the ability to maintain a constant relative humidity and temperature independent of ambient conditions within the electrospinning enclosure was investigated. This was done with the hopes of reducing possible variability introduced by previously uncontrollable changes in ambient conditions associated with the weather.

4.2.2.2 Environmental Effects

The greenhouse temperature and humidity controller controller, terrarium humidifier/fogger, and thermoelectric dehumidifier mentioned previously in Chapter 3, section 3.2.2, as well as a 1500-watt ceramic safety furnace were acquired to control to control humidity and temperature within the electrospinning enclosure. Each were tested separately to determine their ability to alter the ambient enclosure environment. The overall goal of the humidity-controlling equipment was to develop the ability to select a particular relative humidity value and hold the chamber at such a point for the duration of an electrospinning session (approximately 1 hour). The goal of the heater was to hold the chamber at constant temperature above 20 °C.

The fogger could increase the humidity of the enclosed space to 82% from an ambient relative humidity of 25%, while the limit of the dehumidifier's ability was lowering the relative humidity to 45% from 60%. Subsequent dehumidifier tests showed that the humidity can be lowered even farther, however this was only possible at lower starting ambient humidity values. This provided a large range of possible humidity values to test. Additionally, the presence of visible fog during the high humidity tests showed places in which fume hood-induced air flow occurred in the chamber. It was observed that there was little-to-no fog disturbance between the needle tip and mandrel during this test, suggesting that the air flow from the fume hood does not have a large effect on the traveling polymer jet during electrospinning. As detailed previously in Chapter 3, spins were performed at elevated and depressed relative humidity values to investigate the effect of environmental conditions on PLGA electrospun from a chloroform solution,

however the results suggested that there was no significant difference in fiber diameter of the resulting scaffolds (Figure 53).

Unfortunately, the ceramic furnace was unable to consistently heat the enclosed space, possibly due to the relatively large volume of air in the chamber and the fact that the fume hood is constantly pulling cooler air past the walls of the chamber, thus cooling the enclosure and the air within it. Additionally, an onboard temperature sensor turned the furnace off several times during testing before any consistent temperature above standard ambient conditions had been reached, accentuating the furnace's inability to provide a consistent temperature output and further invalidating this heating method.

The method used to measure these changes in environmental conditions was a combination of the aforementioned greenhouse humidity and temperature controller and a generic, battery-operated humidity and temperature monitor that had been used for all previous ambient measurements of the electrospinner in the lab (Figure 67).

Figure 67. Image of the humidifier (top left), controller (top right), and entire setup inside the electrospinning fume hood and enclosure.

When attempting to increase the humidity using the fogger/humidifier it was determined that the sensor of the controller was too sensitive, and that a local increase in humidity would cause readings to spike and the controller to disengage the humidifier,

slowing the process. For this reason, the humidifier was run for several minutes until a significant increase in humidity was displayed on the more gradually-changing monitor, at which point the controller was used to more finely alter the relative humidity without frequently interrupting the humidifier. This technique was used to hold the chamber at 79% RH for Spin 2E from Chapter 3, section 3.3.1. Based on the results described here and in section 3.3.1, it has been shown that an elevated relative humidity can be achieved and held for the time required to spin a scaffold; however, elevated humidity has not been shown to have an effect on electrospun PLGA fiber diameter and makes scaffold removal more difficult.

4.2.3 Scaffold Removal

Four separate treatments were considered for mandrel surface modification with the intention that a smoother surface should improve ease of scaffold removal: polishing mandrels with automotive polishing compound (Meguiar's, G17216), coating mandrels with a Teflon lubricant (DuPont, DNS614101), and fabricating new mandrels from pre-polished, mirror finish 304 stainless steel rod stock (McMaster-Carr, 1256T21). Additionally, some mandrels were also sanded with a finer abrading surface (4000 grit). This was done with the aid of a handheld drill to rotate the mandrel while sandpaper was applied with pressure. In this way, the variations due to individual operator technique were diminished. These treatments were performed once and mandrels were not sanded with 1200 grit paper after treatment, only cleaned with isopropyl alcohol prior to electrospinning. Treatments were scored on a 1-5 scale (1 – worst, 5 – best) on ease of

application, mandrel material compatibility, and most importantly ease of scaffold removal (Table XXIII).

Table 23. Mandrel Surface Finish Treatment Scores

	Auto Polish	Teflon	Pre-Polished	4000 Grit	Control
Ease of Application	3.5	5	-	4	4
Material Compatibility	4	2	-	5	5
Scaffold Removal	5	3	4.5	5	2

Each mandrel was spun with 2 scaffolds for the purposes of this evaluation. The scaffold, including the luminal surface, did not appear noticeably from each other or from scaffolds spun previously different when using direct visual observation and with SEM images. In most respects, all treatments showed a noticeable improvement compared to the 1200 grit sandpaper abrasion control sample.

4.2.3.1 Automotive Polish

The automotive polish treatment was performed as described on the polish container: the polishing compound was applied to a soft cloth and rubbed onto the entirety of the surface of the scaffold until the entire surface had been covered. Because the polishing compound does not seem as abrasive as sandpaper, the application of polish was done with moderate pressure. Although this is not easily quantifiable, polishing until the cloth had been colored by metal particles and the surface of the mandrel appeared consistently smooth and polished. While the compound was originally intended for painted surfaces, it appeared to produce a consistent surface finish on the stainless steel

mandrel as well. Scaffold removal was very easy, requiring almost no force to slide the scaffold from the mandrel.

4.2.3.2 Teflon Spray

The Teflon lubrication was easy to apply; the product was simply sprayed onto the mandrel similar to other spray-on coatings like paint. However, even with careful application the resulting Teflon coating appeared uneven and was visibly altered by physical contact, suggesting that the coating did not adhere to the mandrel surface. Removal of a scaffold from Teflon-treated mandrels was somewhat improved compared to the control, however the force required for removal was not consistent between scaffolds.

4.2.3.3 Pre-Polished

Scaffolds were spun onto prepolished mandrels in a similar manner to all other mandrels tested, however they lacked the spring pin “t” intersection that had been added to all other mandrels used in the BVM lab. Because of the pre-polished nature of the surface finish, it could not be held securely or machined without risk of marring the mandrel surface. The absence of a “t” removed a common point onto which operators hold the mandrel after spinning, resulting in direct handling of scaffolds spun onto the pre-polished mandrels. Removing scaffolds from the pre-polished mandrels was done with noticeable ease, similar to the automotive polish trials.

4.2.3.4 4000 Grit Sanding

The 4000 grit sandpaper treatment was performed by spinning each mandrel in a handheld drill while applying the sandpaper to the spinning mandrel surface; This was done to ensure a consistent surface finish. Scaffolds removal from mandrels polished with 4000 grit sandpaper were done with equivalent ease to that of the mandrels treated with automotive polish.

Of the 4 types of mandrel surface treatments evaluated and compared to the BVM standard of 1200 grit sandpaper polish prior to electrospinning, mandrels treated with either automotive polish or 4000 grit sandpaper provided the easiest, most consistent scaffold removal experience, and were both noticeably better than the control mandrels.

4.3 Discussion

For Aim 3, the BVM lab electrospinning protocol was deconstructed and examined for areas of potential improvement. Several areas were identified at various points in the electrospinning process, and efforts were made to limit confusion and standardize a technique for all operators to follow, to reduce scaffold variability, and to incorporate any knowledge that had previously only existed by word of mouth. Overall, the following aspects of the protocol were clarified or adjusted: the unnecessary PLGA mass precision required in the previous solution mixing protocol, the position of an electrostatically charged wirer relative to the electrospinning jet, the environmental conditions within the electrospinning chamber, and the surface finish of the mandrels

used as the grounded surface in the electrospinning process. These aspects will be reviewed and discussed below.

4.3.1 Solution Mixing

The previous iteration of the electrospinning solution mixing protocol required an unpractically high level of polymer mass-weighing precision, which did nothing to improve scaffold quality or consistency and was obviated by the relatively unprecise solvent measuring technique. The allowed deviation in polymer mass was increased from 0.0001 to 0.004 g, still only 0.5% of the target polymer mass, which translated to a deviation in solution concentration of less than 0.1wt%. By comparison, this difference in overall polymer concentration for the standard 15wt%, 0.7835g of PLGA electrospinning mixture was equivalent to a deviation in measured solvent on the order of tens of μL from the overall target value of 3.0 mL. The new protocol would allow for a range of PLGA masses between 0.7795 and 0.7875 g, easily attainable within a matter of minutes rather than 30 or more. Regarding the imprecision of the current solvent measuring step: it is advised that the BVM lab switch to using syringes with maximum capacities of 3 mL instead of the typical 10 mL syringes; the smaller syringe has more graduations and a greater physical length per mL, allowing for greater solvent-measuring precision.

4.3.2 Electrospinning Setup

The placement of the wire connecting the power supply to the needle and polymer solution was suspected to be the cause of a disparity in polymer coverage between the

proximal and distal portions of the mandrel during electrospinning. The assumption was that the current running through the wire generated a magnetic field strong enough to interfere with the polymer jet as it traveled from the needle tip to the grounded mandrel, repelling it from the distal side where the wire was suspended and creating a noticeable difference in coverage. This was tested by moving the wire from inside the electrospinning enclosure to under the syringe pump, directly in line with the needle such that there was no portion of the wire that was located on either side of the needle. Scaffolds were spun with each wire orientation and the difference in scaffold wall thickness between the distal and proximal ends of each scaffolds were evaluated. This test showed that the old wire placement did in fact produce scaffolds with a significant difference in wall thickness, while the new wire placement produced scaffolds of equal thickness throughout. This new wire placement was taught to all electrospinning operators and included in the revised BVM lab electrospinning protocol to prevent any future instances of this disparity.

4.3.3 Environmental Conditions

It was determined that the electrospinning enclosure can be modified to increase the relative humidity inside to approximately 80% using a simple terrarium humidifier and controller. Although a previous experiment discussed in section 3.3.1 in this thesis determined that there was no significant difference in average fiber diameter between high and low humidity values on PLGA scaffolds spun in the electrospinning lab, the steps required to implement these additional components can be easily incorporated into the current electrospinning protocol with little modification. Because the results of the

humidity test in section 3.3.1 suggest that a lower humidity is preferable for scaffold removal purposes, the only protocol modification in this regard has been to keep to the thermoelectric dehumidifier on inside the electrospinning chamber between spins to reduce ambient RH (Appendix G,H). As discussed previously, there are multiple polymer-solvent combinations that have been shown to change dramatically with changes in relative humidity, and it is possible that the BVM lab may use other materials more susceptible to changes in humidity in the future^{101–103,139}.

However, the attempts to regulate the environment of the electrospinning enclosure were not entirely successful, particularly with regards to dehumidifying and heating the chamber. This could be attributed to several factors: The equipment used for these tasks was intended for recreational use in a small room, was not constructed to reach specific metrics related to its function. The fact that the electrospinning enclosure is not hermetically sealed and is located inside a fume hood made the task of regulating the environment even more challenging for these devices. Creating a sealed enclosure and mitigating the effect of the outside environment will be key for future endeavors into regulating the electrospinning environment.

4.3.4 Scaffold Removal

Mandrels with several different surface treatments were used as substrates in BVM lab electrospinning to characterize their ability to reduce the issue of scaffolds sticking to their mandrel. The previous electrospinning protocol called for mandrels to be

hand-polished and cleaned with 1200 grit sandpaper and isopropyl alcohol directly before electrospinning to create a standardized surface for all spins, however scaffolds were still difficult to remove. Scaffolds used in the experiment were treated with either automotive polish, 4000 grit sandpaper, a PTFE spray coating, or were purchased pre-polished from McMaster-Carr. It was determined that the 4000 grit sandpaper treatment was easiest to apply, was compatible with the 303 stainless steel mandrel material, and provided the smoothest scaffold removal, followed closely by the two types of polished mandrel. While it was intuited that the surface roughness of each mandrel subjected to an abrasive treatment was being decreased, this was not assessed quantitatively; use of a profilometer may yield precise roughness values that can be correlated to mandrel performance. Additionally, it may also be possible to produce a more uniform surface finish by sputter coating mandrels with materials that will result in lower friction coefficient values with PLGA and other electrospinning polymers.

While the pre-polished mandrel performed nearly as well as the 4000 grit mandrel, it was difficult to remove from the electrospinner at times, and required the scaffold to be handled more to release it from the electrospinning collector. Most mandrels used in the BVM lab are purchased as cylindrical rod stock and are machined to accommodate a spring pin, forming a “t” intersection that interfaces with the electrospinning. After the pin is pressed in place the mandrel can be polished and used normally. The pre-polished mandrel, however, could not easily be held in a vice to drill a hole for the spring pin without marring the polished surface, and so they were held in the

electrospinner under a compressive force introduced by a spring-loaded component (Figure 68).

Figure 68. Example of a mandrel loaded into the electrospinning collector with the spring loaded-barrel on the distal end under no load (top) and under compression (bottom)

This wedged the mandrel into the collector and required a significant application of force to remove, sometimes disturbing the recently spun scaffold in the process. The lab did have access to “t” adapters fabricated from miniature drill chuck parts, used previously with 1 mm diameter mandrels, however they reduce the amount of exposed mandrel considerably and thus reduce the amount of useable scaffold generated from electrospinning¹¹⁴.

Surprisingly, the PTFE-coated mandrel performed only somewhat better than the mandrels sanded per the standard protocol. The PTFE coating was a commercially available spray coating advertised as a non-stick dry-film lubricant that bonds to the surface it is sprayed upon. However, it was observed that directly after spraying the coating on two mandrels that they both had an uneven, cloudy coating that was easily rubbed away even after they were left to dry. This suggests that the lubricant did not properly adhere to the mandrel surface, and that the product is intended as a lubricant more so than a method to apply a permanent coating. Even if the PTFE-coated mandrels had allowed scaffolds to be removed with little to no effort, it was possible that the coating may leach into the scaffold and potentially disrupt or prevent cell adhesion and proliferation, invalidating and BVM setup results it affected. For these reasons the PTFE coating was not pursued further.

Prior to these experiments the force required to remove a scaffold from a mandrel seemed mostly arbitrary, however operators noticed that the average force required for removal seemed to increase during instances of high electrospinning enclosure humidity. This was corroborated by observations in Chapter 3, in which the high humidity scaffold could not be removed from the mandrel through any means aside from being cut off in sections. This effect appeared to diminish once all electrospinning was performed on 4000 grit-sanded mandrels, however over time this trend reappeared. As mandrels are used and marred by general use and contact with the scalpel blades used to remove scaffolds, they also need occasional re-polishing to retain their positive effect on scaffold removal. It was decided that scaffolds would not be polished prior to every spin to reduce

the possible presence of metallic particles on the luminal surface of the scaffold. Thus, a clause in the updated electrospinning protocol was included to specify a re-polishing frequency of every 3 months or when scaffold removal becomes noticeably more difficult.

4.4 Conclusion

Chapter 4 was focused on fulfilling Aim 3 of this thesis: to investigate inconsistencies within the electrospinning protocol and setup and attempt to mitigate them. Several aspects of the process were considered including the polymer solution mixing protocol, the placement of the charge-carrying wire and the effect it had on scaffold properties, the ability to control the electrospinning enclosure environmental conditions, and the relative ease with which a scaffold could be removed from a mandrel after electrospinning. Alterations to the mixing and electrospinning protocols were made as necessary to clearly indicate new, more appropriate polymer mixing procedures and placement of electrospinner components; the updated mixing and electrospinning protocols are available in Appendix G and H, respectively, while the old protocols are available in Appendix D and E. Additionally, it was determined that the nature of the electrospinning enclosure caused difficulty in regulating temperature and humidity at this time, and that a new chamber should be considered to aid in improving environmental parameter consistency. Finally, 4 mandrel surface finishes were compared to the standard mandrel preparation steps to improve the consistency and overall ease of scaffold removal; it was determined that mandrels polished with 4000 grit sandpaper as needed (compared to polishing every spin, reducing the possibility of metal particulates on the

scaffold's luminal surface) would be the most favorable option, and was also incorporated into the electrospinning protocol.

After improving and introducing characterization techniques to evaluate fiber and pore size to fulfill Aim 1, investigating parameters to decrease fiber diameter and pore size for Aim 2, and optimizing the electrospinning protocol for Aim 3, the next goal was to further improve the scaffold product by addressing another issue vexing researchers in the lab. Specifically, the next chapter will focus on of unpredictable scaffold shrinkage that began occurring during sterilization and conditioning steps. This shrinkage manifested in contractions in length and diameter of scaffolds in an unpredictable, uneven manner. These changes in dimension meant that results from the testing of vascular devices designed for a specific vessel geometry could be inaccurate, severely limiting the capabilities of the BVM setup. The following chapter describes testing to elucidate the underlying reason for the shrinkage and experiments to mitigate this effect.

5. INVESTIGATING AND MITIGATING SCAFFOLD SHRINKAGE

5.1 Introduction

An unfortunate and perplexing phenomenon has been observed in the BVM lab for some time now: the unpredictable shrinkage of scaffolds during the early stages of a BVM setup. Once secured to luer lock fittings, scaffolds undergo a series of rinses in 70% ethanol, Dulbecco's dication-free phosphate buffered saline (DCF-PBS), and media, to flush out contaminants and kill any microbes present on the scaffold surface that may interfere with cell adhesion and viability. After sterilization, the scaffold is inserted into the bioreactor setup which is placed inside a cell culture incubator for approximately 12 hours while conditioning media is continuously flowed transmurally through the scaffold and BVM system via a peristaltic roller pump (Figure 69).

Figure 69. Sterilization of scaffolds using an ethanol submersion (top) and BVM setup images (bottom) showing the path of media flow through the scaffold and bioreactor via peristaltic pumping while in incubation conditions.

Shrinkage in length and overall diameter has been observed during and after the ethanol sterilization stage; furthermore, because the bioreactor holds the scaffold to a particular length within the system, shrinkage during conditioning is limited to changes in diameter. This shrinkage is not consistent across the length of the scaffold, and will unevenly change the shape of the construct in ways that inhibit accurate assessment and device deployment and evaluation. Chapter 5 is dedicated to the 4th aim of this thesis: Improving scaffold consistency and use by understanding and reducing PLGA scaffold

shrinkage. The first step in fulfilling this aim was to review polymer science and electrospinning-specific literature to find other instances of this phenomenon as well as possible causes and solutions. Next, two different solutions were evaluated experimentally by their ability to mitigate scaffold shrinkage.

Shrinking of electrospun scaffolds of several polymeric materials has been documented previously: Ru et al. reported shrinkage values of 75% and above when submerging a circular electrospun mat of 50:50 PLGA into PBS at 37°C for 24 hours in preparation for culturing human skin keratinocytes. This issue was mitigated by holding the PLGA mat in the desired shape with an auxiliary support structure, physically preventing the scaffold from shrinking⁶⁸. Jose et al. electrospun nanocomposite scaffolds for bone tissue engineering from 85:15 PLGA and nanoparticles of hydroxyapatite (HA) and noted initial scaffold shrinkages of 9-49% after submersion in PBS at 37°C for 1 hour to simulate bodily conditions. Furthermore, it was confirmed that the shrinkage found by Jose et al. was due to the thermal effect of holding the scaffold above room temperature rather than any plasticizing effects of water by sealing the scaffold in aluminum foil and heating it on a hot plate to 37°C for 1 hour. This idea was reinforced through use of differential scanning calorimetry (DSC), which suggested that the effective glass transition temperature of the electrospun PLGA had decreased from stock PLGA values of 43°C to between 32 and 37°C depending on HA content⁶³.

The results produced by Zong et al. support these findings, as they also experienced significant shrinkage in several compositions of PLGA including 75:25 in

aqueous incubation conditions, and confirmed that heat rather than interaction with water was the main cause of shrinkage for 75:25 PLGA through similar dry heating methods⁶⁴. Finally, Tseng et al. described a stress-relieving heat treatment performed on a PLGA-containing shape memory polymer slightly above glass transition temperature to eliminate any unwanted changes in shape due to shrinkage. Their polymer was embedded in a reversible hydrogel to hold the polymer structure in place during heating to prevent any changes in shape or fiber alignment. These literature sources confirm that electrospun material, and PLGA specifically, have exhibited noticeable shrinkage in previous experiments. They have also shown that this shrinkage can be triggered by increases in temperature, and that electrospun structures experience a depression in T_g as compared to their bulk state. Finally, stress-relief methods were described in which the macroscopic scaffold structure was held in place and ultimately retained.

Based on these findings, attempts were made to characterize the differences between stock PLGA and PLGA scaffolds spun in the BVM lab and to mitigate scaffold shrinkage through multiple stress-relieving techniques on PLGA scaffolds produced within the BVM lab.

5.2 Methods and Results

First, stock PLGA pellets and electrospun PLGA were evaluated using differential scanning calorimetry (DSC) to determine any changes in effective glass transition temperature. Then electrospun PLGA scaffolds were treated to relieve internal stress and

allow the polymer chains to reposition. Treated and untreated scaffolds were then subjected to a 30-minute ethanol soak and 12 hour conditioning-mimicking step at elevated temperature to evaluate any differences in shrinkage.

5.2.1 Differential Scanning Calorimetry

To confirm that electrospinning PLGA noticeably lowers its effective T_g below that of the stock material and below the ambient temperature necessary for cell culture, samples of PLGA in both pellet and electrospun states were analyzed using differential scanning calorimetry (DSC). DSC was used to heat each sample alongside an aluminum control sample and record the energy input required to keep both samples at the same temperature. Fluctuations in energy input (visualized as significant changes in slope of the green line in Figures 69 and 70) suggested a transformation of some sort, such as instances of significant changes in crystallinity, melting and solidifying, or a transition from glassy to rubbery states. Each sample was ramped up from approximately 20°C to well above the glass transition temperature range of 50-55°C provided by the manufacturer at a rate of 10°C/minute (Figure 70 and 71)⁶². It should be noted that measurements for crystallinity and determination of crystallization temperature were not performed due the amorphous nature of 75:25 PLGA⁶².

Figure 70. Full DSC output for as-obtained 75:25 PLGA pellets (top) and the glass transition region (bottom) of the curve.

Figure 71. Full DSC output for electrospun 75:25 PLGA (top) and the glass transition region (bottom) of the curve.

It was observed that the glass transition temperature of stock PLGA pellets was approximately 47°C, slightly lower than the 50-55°C range provided by the manufacturer. Additionally, the glass transition temperature of electrospun PLGA was determined to be 34.26°C, noticeably lower than both stock PLGA and the temperature experienced during BVM setup, 37°C. This supported the assumption that electrospinning was increasing the internal stress within the polymer chains. A lower T_g represented the fact that comparatively less energy must be added to the system to achieve the same transition, suggesting that the difference in energy is stored within the stressed polymer chains; transitioning to a rubbery state was more favorable at lower temperatures in PLGA after electrospinning⁶⁰. Additionally, the depressed T_g of the electrospun sample falls below the incubation temperature of the BVM setup, allowing the transformation to continue during the scaffold conditioning phase. This means that the 37 °C conditions of the incubator, a necessary condition for BVM setups, is responsible for triggering scaffold shrinkage in some capacity.

5.2.2 Stress Relief Treatments

Scaffolds were spun per the newly updated BVM lab electrospinning protocol (Appendix H) for the purposes of testing multiple stress-relieving treatments. During this time in the BVM lab, large supplies of chloroform used for PLGA electrospinning had expired and were in the process of being phased out in favor of new chloroform. The issue of shrinkage was thought to possibly be exacerbated by the use of the expired chloroform, and so scaffolds were spun with both new and old chloroform to directly compare their shrinkage characteristics.

As-spun scaffolds were divided into several pieces so samples could be retained for imaging or used as a control (Table XXIV). To retain the macroscopic cylindrical tube structure of the scaffold in the intended dimensions, scaffolds were left on their mandrels during all treatments (Figure 72). In this way, polymer chains could relax and reorder towards a more thermodynamically stable orientation without experiencing large-scale shrinkage. Stress-relieving treatments included either a 3-hour heat treatment at 55°C or a 1 hour soak in 70% ethanol (Figure 72).

Heat treatment above the 75:25 PLGA glass transition temperature was selected based on previous literature results that described similar shrinkage issues and based on guidance provided by Dr. Philip Costanzo of the Cal Poly Chemistry Department^{63,68,69}. A 70% ethanol soak was attempted as a stress-relieving technique because prolonged exposure to ethanol had previously shown to increase polymer chain mobility in PLGA scaffolds in the BVM lab when unexpected scaffold shrinkage occurred. Because deformation had been shown to occur at or before 30 minutes, 1 hour of ethanol exposure for stress relief was selected to ensure polymer chain relaxation.

Table 24. Descriptions for Scaffold Sections Spun for Stress-Relief

Scaffold section	Description
Stress Relief for SEM	Underwent stress relief and then imaged to observed any morphological differences caused by stress relief treatment.
Control Sample	As-spun section used for shrinkage comparison; underwent an ethanol soak and 12-hour conditioning step to induce shrinkage
Stress Relief and BVM Steps	Subjected to stress-relief treatment (either heat or ethanol) and then a sterilization-mimicking ethanol soak followed by a conditioning step to evaluate any mitigation of shrinkage.
As-Spun SEM Sample	SEM sample from the as-spun scaffold; acted as a control for the “Stress Relief for SEM” to compare against.

Figure 72. Scaffold divided for stress relieving experiments (top), scaffold sections prepared for heat treatment (bottom left) and for ethanol treatment (bottom right).

After either a heat or ethanol stress-relieving treatment, each treated scaffold section along with a counterpart control sample from the same as-electrospun structure were then subjected to conditions that mimicked the BVM setup environment.

5.2.3 Shrinkage Testing

First, the treated and control scaffold sections were secured to luer lock fittings and submerged in a 70% ethanol solution at room temperature for 30 minutes to mimic the scaffold sterilization step. Scaffolds were then subjected to a 12-hour heat treatment at 37°C to mimic the conditioning step of the BVM setup. These steps were specifically selected because they have been observed to cause scaffold shrinkage in past bioreactor setups (Figure 73). Length and diameter measurements were made before and after all treatments, and SEM images of portions of each scaffold were taken to observe any changes in fiber morphology.

Figure 73. Scaffold sterilization treatment (left) and a heat treatment step inside a low-temperature furnace at 37°C (right) to mimic the BVM conditioning environment.

5.2.4 Shrinkage Results

Two scaffold sections of each group (heat treated, ethanol treated, and control) were tested; the results of their dimensional changes before and after shrinkage testing are summarized in Table XXV. SEM image comparisons between as-spun PLGA scaffold sections and sections that underwent stress relief treatments showed that some smaller fibers on the luminal surface of treated sections appeared to lose tension or partially combine with larger underlying fibers (Figure 74). The presence of white, amorphous particles that appeared to fill some spaces between fibers was also noticed on scaffolds post-sterilization. These tests were not done in a sterile environment, and either the 70% ethanol solution or container may have been contaminated as a result. Additionally, the as-spun control sections that experienced the sterilization and conditioning steps were observed to have noticeable dimensional changes in their macroscopic structure (Figure 75).

Table 25. Average Decrease in Scaffold Length and Diameter After Sterilization and Conditioning

Treatment	Chloroform Type	Length		Diameter	
		Difference (%)	Std. Dev. (%)	Difference (%)	Std. Dev. (%)
Heat Treated	Old	5.13	6.37	4.35	1.87
	New	0.77	4.95	1.18	2.98
Ethanol Treated	Old	5.20	-	5.06	-
	New	1.41	0.34	0.59	0.72
Control	Old	15.98	0.16	10.39	0.59
	New	8.53	3.28	0.78	5.25

Figure 74. SEM images of scaffolds prior to heat treatment (top left), after heat treatment (top right), and after sterilization (bottom left) and conditioning-mimicking step (bottom right). The white artifacts are of unknown origin.

Figure 75. Scaffold section prepared for sterilization and conditioning (top) and a comparison of treated and untreated scaffold sections after sterilization and conditioning (bottom).

The values presented in Table XXV were plotted to further illustrate noticeable differences between values and allow for a direct, visual comparison of results (Figure 76).

Figure 76. Plot showing average difference in scaffold length and diameter between stress-relieved and control samples after BVM sterilization and conditioning treatments.

It was immediately apparent that the expired chloroform had a noticeable effect on the results of all groups. Additionally, it was also apparent that both stress-relief treatments noticeably reduced shrinkage compared to control groups by approximately the same amount. This suggested that both heat treatment and ethanol exposure have the same effect on the polymer chain realignment and to the same extent.

Additionally, DSC scans were made of electrospun PLGA scaffolds after stress-relieving treatments to observe the degree to which the apparent T_g returned to that of stock 75:25 PLGA. It was found that T_g had increased from the 34.26 °C value of as-spun PLGA scaffold to 43.95 and 47.78 °C for heat and ethanol-treated, respectively (Figure 77 and 78). These values were comparable to the T_g value of 46.94 °C measured for stock

PLGA pellets, suggesting that the treatments successfully allowed the polymer chains to realign to a more stable state.

Figure 77. DSC curve (top) and glass transition area (bottom) of electrospun PLGA scaffolds after heat treatment.

Figure 78. DSC curve (top) and glass transition area (bottom) of electrospun PLGA scaffolds after ethanol treatment.

5.3 Discussion

It has been observed that electrospun PLGA scaffolds sometimes shrink during the first few steps of the BVM setup, specifically during exposure to ethanol and elevated temperatures. This is an issue because it changes the dimensions of the scaffold, limiting its ability to accurately assess cell behavior or device deployment in a vessel. Aim 4 set out to understand and mitigate this shrinkage problem.

5.3.1 Reasons for Shrinkage

The process of electrospinning elongates the polymer jet during its travel between the needle tip and the mandrel. This elongation also results in polymer chain alignment, in which a higher-than-equilibrium number of polymer chains also align unidirectionally due to the formation of high-aspect ratio polymer fibers. Solvent evaporation occurs before the polymer chains can naturally return to a randomized orientation, freezing them in a metastable state⁶³. The Aligned polymer chains of a fibrous, electrospun structure are in a higher energy state than when the chains are randomly arranged, which manifests as a decrease in apparent glass transition temperature of the scaffold when analyzed with a DSC. In reality, the glass transition temperature is inherent to the material itself and is not actually changing during electrospinning, however the forces that are attempting to return the polymer chains to their randomly arranged, non-aligned state are contributing to the total amount of energy needed for chains to un-align. Therefore, this chain movement occurs with less external energy input, at a lower temperature. This means that conditions which would normally not significantly alter bulk PLGA can cause noticeable shrinkage in electrospun structures when exposed to heat or ethanol.

5.3.2 Effect of Stress-Relief

Exposure to both ethanol and elevated temperatures have been shown to cause scaffold shrinkage in the sterilization and conditioning steps of the BVM setup procedure. Ethanol is a poor solvent to PLGA but increases chain mobility enough to initiate chain rearrangement. As shown by the DSC curves of electrospun PLGA, the 37°C experienced during the conditioning phase is at or above the seemingly-depressed T_g value of electrospun PLGA, meaning it provides enough energy to initiate chain realignment as well. Because of this, both ethanol and heat exposure were effective as stress-relief treatments. By holding the macroscopic shape of the scaffold constant, treating scaffolds in these conditions allowed for chain realignment while maintaining the tubular scaffold shape in the dimensions necessary to interface with the BVM system. As was shown by the shrinkage data, scaffolds that underwent either ethanol or heat treatments prior to sterilization and conditioning shrunk noticeably less than as-spun control samples.

5.3.3 Difference Between Chloroform

In addition to the noticeable difference in scaffold shrinkage with stress-relief compared to control samples, scaffolds spun with expired chloroform tended to shrink in every group. This may be due to improper handling of the chloroform: Instead of temporarily penetrating the gas-impermeable membrane to extract chloroform from the container, operators had opened the entire bottle to the ambient laboratory environment

regularly. Chloroform is known to be hygroscopic to some degree, and it is assumed that water was absorbed from the regular exchange of gas that took place during improper handling. In addition to altering the electrical properties of the electrospinning solution, this retained water, which is less volatile than chloroform, may have been retained until exposure to heat or ethanol. The escape of water during these stages may have contributed to the shrinkage experienced by scaffolds spun with the improperly handled, expired chloroform and exacerbated instances of shrinkage during BVM setups. This suggests that the proper techniques for handling chloroform directly impact the results of electrospinning in the BVM lab. The proper chloroform handling procedures are documented in the revised electrospinning protocol, Appendix G.

5.4 Conclusion

It was assumed that the cause of scaffold shrinkage was due to the formation of a metastable phase in which aligned polymer chains were held in a higher-than-equilibrium energy state after electrospinning due to solvent evaporation. This was confirmed through use of differential scanning calorimetry, in which the apparent glass transition temperature of the electrospun material was noticeably lower than that of stock PLGA. Stress relieving treatments of either heat or ethanol exposure were shown to reduce scaffold shrinkage noticeably compared to a control, and a difference between new and old, improperly handled chloroform was also observed. It is recommended that all scaffolds are heat or ethanol treated prior to use in the BVM bioreactor to mitigate any unwanted shrinkage or other changes in scaffold geometry. Based on observations made during the stress-relieving process, it is advised that heat treating be pursued over ethanol

treatment: stress-relieving in ethanol uses a lab consumables each time it is performed, whereas the low-temperature oven simply uses electricity. Additionally, after ethanol treatment the scaffolds must dry before they are handled, and because they presumably dry on the outer surface first and the inner surface last, it is difficult to determine the time at which ethanol-treated scaffolds can be handled without fear of damaging them. Heat treatment can be done in large batches if necessary, and scaffolds cool within minutes of removal from the oven.

6. INVESTIGATION OF A FLEXIBLE ELECTROSPINNING MATERIAL

6.1 Introduction

Following extensive work with PLGA electrospinning and scaffold characterization and post-processing, as described in the previous three aims and four chapters, the final aim of this thesis took a step back to determine if PLGA is the best polymer to use. Prior to the use of electrospinning in the BVM lab, lengths of ePTFE tubing were obtained and used as scaffolds^{7,70}. While ePTFE is consistent and reliable, it has mechanical properties noticeably different than those of native vessels and can lead to thrombosis and hyperplasia. While these were not immediate concerns due to the use of cell media rather than blood in BVM lab bioreactors, ePTFE was also considered too expensive to be a sustainable scaffold material for the lab, and could not be modified or customized to fit other needs outside the basic BVM setup⁷. For these reasons, electrospinning was sought to reduce cost and allow for more physiologically favorable polymers to be used for scaffold fabrication and to allow for increased customizability. Since 2009, PLGA has been the longstanding polymer of choice for the vast majority of *in vitro* cell observation and device testing within in the BVM lab^{6,70,71,114}.

Although the most commonly electrospun material in the BVM lab is 75:25 PLGA, the initial characterization and qualification of the BVM electrospinner was done with 90:10 P(LLA-CL), a copolymer of poly(l-lactide) and poly(ϵ -caprolactone)⁷. PLGA was selected shortly after due to superior endothelial cell response reported in published literature, prior instances of successful electrospinning, controlled degradation,

sterilizability, mechanical properties similar to that of a native vessel, low cost, and polymer availability⁷⁰. While PLGA has served adequately as the polymer of choice for BVM scaffolds for 8 years, there are certain material characteristics that could be improved. While PLGA may have “true” mechanical properties similar to those of native blood vessels, in reality the scaffolds are spun to such a thickness that a rigid tube is formed, due to concerns that thinner scaffolds will rip or allow cells to pass through. The thick-walled PLGA structure does not allow for much elastic bending, unlike a native vessel, and thus all cell seeding and device testing is done with straight scaffolds. One of the goals of the BVM lab is to test devices in more tortuous conditions, and the rigidity of the current PLGA scaffolds do not allow for any substantial changes in orientation (Figure 79)⁸. In addition, even bending the scaffold into the chamber for a straight-vessel set up can be challenging. The thick, inflexible walls of the current scaffolds may also have some effect on cell behavior as a result of not properly mimicking blood vessel mechanical properties.

Figure 79. Complex scaffold configurations designed by previous BVM lab researcher Dalton Chavez using ePTFE⁸. Electrospun PLGA would not be able to be formed into any of these geometries due to its lack of elasticity.

Additionally, through anecdotal lab experience, there appears to be some amount of scaffold degradation occurring. While scaffolds appear malleable and flexible directly in the days immediately following electrospinning, they eventually become stiff and more prone to cracking as time goes on. While this degradation has only been shown to manifest in the BVM lab in some instances when measuring ultimate tensile strength, the ramifications of degradation on the microscale may have implications for cell behavior

during BVM setups and device testing¹⁴⁰. Finally, the cost of PLGA has risen over time to \$54 per gram (and thus nearly \$50 per scaffold) and is quite expensive compared to other biopolymers used in tissue engineering and specifically electrospinning. This limits the amount of experimentation and experimental replicates produced in the lab, which results in less statistical confidence when observing the effect of parameter changes on the electrospinning process. To facilitate more experimentation regarding device positioning and orientation as well as electrospinning-specific research, exploring a flexible, low-cost PLGA alternative was the goal of the fifth and final aim of this thesis.

6.1.1 Flexible Polymer Use in Vascular Tissue Engineering

The mismatch in mechanical properties between native blood vessels and ePTFE, as well as several other biopolymers, has necessitated the investigation of materials with which compliant tubes can be fabricated for the purposes of vascular tissue engineering. While there are countless polymers and copolymers that have been synthesized and tested for this very use, this thesis focused on three materials specifically: Poly- ϵ -caprolactone and two aliphatic, polycarbonate-based thermoplastic polyurethanes. All three were selected based on their flexibility and on literature sources that describe their successful use in electrospinning applications for tissue engineering. The materials selection process that led to the selection of these three materials will be covered extensively in the following sections of this chapter, along with subsequent electrospinning and flexibility testing of ePTFE, PLGA, PCL, and tPU to provide a set of initial characterizations for future lab use.

6.2 Methods and Results

Several flexible materials suitable for electrospinning for tissue engineering were identified, evaluated, and ranked. Ultimately PCL and two variations of a polycarbonate-based tPU were selected for further testing by scaffold fabrication in the BVM lab electrospinning setup. Scaffold morphologies were compared to those of typical PLGA scaffolds to establish a baseline for future use. Finally, sections of ePTFE, PLGA, and flexible polymer scaffolds were tested for flexibility and kink resistance as per ISO 7198. The materials selection process, electrospinning experimental factors and results, and specifics and results of kink testing will be covered in detail in the following sections.

6.2.1 Materials Selection

To select the most appropriate flexible polymers for electrospinning as a comparison for current PLGA scaffolds, 3 criteria were considered: 1). Material flexibility, measured by elastic modulus, 2). Electrospinability, evaluated by the presence of literature covering the process of electrospinning a given material, specific methods used and their similarity to the BVM lab electrospinning setup, and the results observed, and 3). Cost of the material per gram. Additional consideration was given to degradation, sterilizability, and evidence that a material had been successfully used for vascular tissue engineering. Specifically, the material could not undergo significant degradation on the order of a few months or less in environments that mimic bodily conditions or in a typical laboratory setting. Additionally, the material must withstand exposure to a 70% ethanol solution for the purposes of sterilizing in preparation for BVM bioreactor setup, or have a similar, proven sterilization method (Table XXVI).

Table 26. Materials Selection Criteria for Flexible Polymer Electrospinning

Primary Criteria	Material Flexibility Electrospinability Cost
Secondary Considerations	Degradation Sterilization Use in Vascular Tissue Engineering

6.2.1.1 Material Flexibility

Initial materials selection was performed through use of the CES Bioengineering EduPack materials selection software (Granta, 2017). Materials were limited to natural materials and polymers considered biocompatible based on CES data and plotted on a logarithmic scale of Young's modulus (Figure 80).

Figure 80. Plot of Young's modulus of several polymers used in biomedical application⁵⁷.

Unfortunately, ePTFE was not available in the material database, however the presence of standard PTFE showed that it exhibits a lower modulus compared to PLGA. This relative difference in elastic modulus values was corroborated by material property results reported in a previous thesis, in which ePTFE and PLGA displayed values of 7.80 and 13.25 MPa respectively when full tubular scaffolds were mechanically tested¹⁴¹. The polymers that are reported to have lower Young's modulus values compared to PLGA in Figure 80 include: Acrylonitrile butadiene styrene (ABS), polystyrene (PS), thermoplastic polyurethane (tPU), polypropylene (PP), Nylon 11 (PA-11), standard and ultra-high molecular weight polyethylene (PE and UHMWPE), collagen, polytetrafluoroethylene (PTFE), polycaprolactone (PCL), ethylene vinyl acetate (EVA), medical grade silicone, elastomeric polyurethane (ePU), and natural rubber (NR). As a reference value, CES listed the modulus value of native blood vessel tissue as 0.0008 to 0.0015 GPa⁵⁷.

This preliminary materials selection phase was simply used to create a short list of potential flexible polymers for future research. Next, electrospinning literature publications for each polymer with modulus values below that of PLGA were examined to provide insight into the feasibility of using said polymers as constructs for blood vessel tissue engineering.

6.2.1.2 Flexible Polymer Electrospinning Literature Review

All polymers that passed the CES biocompatibility limit and were found to have lower Young's modulus values compared to PLGA were investigated further for the presence of previous electrospinning literature:

Acrylonitrile Butadiene Styrene (ABS): Although the reported Young's modulus value for ABS is lower than that of PLGA, there was no easily identifiable literature suggesting the use of ABS as a material for either electrospinning or tissue engineering. A technique similar to electrospinning referred to as ForcespinningTM, in which centrifugal force rather than electrostatic force is used to form nanofibers, has been used to form nano- and microfibers of several materials including ABS, however the specific details of fiber morphology, size, and application were not reported¹⁴².

Polystyrene (PS): Polystyrene is often used as a reference material for calibrating several types of materials characterization tools, and has been used as a sort of reference material for investigating trends in electrospinning as well^{105,143,144}. And while polystyrene is often the material of choice for many cell culture applications, there existed little if any published literature about the efficacy of electrospun PS as a scaffold for a tissue engineered construct, specifically for vascular purposes or otherwise⁸².

Thermoplastic Polyurethane (tPU): Several types of thermoplastic polyurethane have been used in electrospinning applications, including multiple as either implanted prosthesis or tissue engineered scaffolds. Average fiber diameter results reported rival

those generated within the BVM lab with PLGA, and favorable cell proliferation characteristics made tPU an attractive option to pursue further^{18,145–148}.

Polypropylene (PP): Polypropylene is used in several disposable medical device applications, such as syringes, medical drapes, gowns, sutures, trays, and other similar items¹⁴⁶. However, there were relatively few publications discussing its use as a material used for extended cell culture techniques and for tissue engineering. The few electrospinning articles in circulation detail the fact that PP can be spun either dissolved in solution or from a heat-assisted dissolution electrospinning setup^{149,150}. The effect of polypropylene microfibers of varying sizes on subcutaneous fibrous capsule formation was investigated, however samples were simply implanted and measured for capsule thickness; no cell adhesion or proliferation was noted¹⁵¹.

Nylon 11 (PA-11): Nylon 11 is relatively uncommon as an electrospinning polymer and was not found in much if any tissue engineering literature as scaffold for cell proliferation. Dhanalakshmi et al. characterized the electrospinning of nylon 11 within the context of it being a biocompatible polymer, however they did not investigate the extend of this biocompatibility after electrospinning¹⁵². Additionally, the prospect of using concentrated formic acid as a component of constant scaffold production was another disadvantage of using Nylon 11.

Polyethylene (PE): Both PE and UHMWPE are common, chemically resistant polymers used in medical applications ranging from packaging, films, and pouches to

wear-resistant surfaces for orthopaedic implants such as hip and knee arthroplasty¹⁴⁶. However, there was little evidence of electrospinning literature for either material, especially within a biomedical or tissue engineering context. The information that was available suggested that electrospinning of PE is only possible through a melt process or melt-assisted dissolution process by dissolving the polymer in xylene heated in an oil bath. Average fiber diameters achieved from these methods ranged from 5.4 to 32 μm and 2 to 7 μm , respectively^{80,153}.

Collagen: Collagen is one of the most abundant components in mammalian connective tissue, and so it is reasonable that several types of collagen have been used extensively for a wide range of tissue engineering applications including guides for neural migration, bladder augmentation, bone-mimicking implantable material, and cell-based cartilage lesion therapies^{48,154–156}. Additionally, collagen has been cited several times as a material used in electrospinning: Collagen has been electrospun as the sole scaffold material and combined with materials like PCL, polyethylene oxide (PEO), and polydioxanone (PDO)^{157–159}. Additionally, collagen has been incorporated in scaffolds of other synthetic materials such as Dacron to provide an amount of bioactivity and promote ingrowth of native tissue in cardiovascular grafts¹⁶⁰. Specifically, electrospinning collagen has been found to produce fibers with average diameter between 200 and 600 nm and the resulting scaffold has been shown to successfully culture human keratinocytes and SMCs¹⁶¹.

Polytetrafluoroethylene (PTFE): The extreme chemical resistance of PTFE is well known, and posed a problem as the primary method of polymer preparation for electrospinning involves dissolution in a solvent. Accordingly, reports of electrospinning with PTFE are not common, and those who have done so successfully incorporated several additional mixing and sintering steps to properly use an emulsified form of PTFE, only to have produced a scaffold with altered morphologies and modulus values of 30.7 ± 2.8 MPa, much stiffer than both the ePTFE tubing and PLGA scaffold tested previously in the BVM lab^{141,162,163}.

Polycaprolactone (PCL): Prior literature of PCL as a flexible electrospinning polymer for tissue engineering applications has provided proof of high cell proliferation efficacy in multiple contexts as well as scaffold morphology information that suggested PCL will create scaffolds of equal or smaller average fiber diameter than the current PLGA scaffolds produced in the BVM lab^{20,37,164–166}.

Ethylene Vinyl Acetate (EVA): - Ethylene vinyl acetate, or poly(ethylene-co-vinyl acetate) (PEVA) is a copolymer of ethylene and vinyl acetate that can exist as either a thermoplastic, thermoplastic elastomer, or a rubber depending on the relative amounts of each constituent polymer¹⁶⁷. PEVA is used in a wide range of applications, including automotive, filtration, surfing equipment, and biomedical, specifically in drug release settings¹⁶⁸. There has been little research into PEVA electrospinning, and those who have attempted the process have done so for drug delivery applications in which the material

was intended to degrade on a relatively short timescale; At the time of writing there were no published sources for tissue engineering work with electrospun PEVA. For these reasons PEVA did not appear suitable for use as an electrospun flexible polymer for BVM setup use.

Silicone (Medical Grade): Electrospinning research with silicone is not common, especially for applications in tissue engineering or biomedical engineering in general¹⁶⁹. Results that do exist for silicone electrospinning have shown fiber diameters of approximately 20 μm , well above the desired range of the BVM lab¹⁷⁰.

Elastomeric Polyurethane (ePU): The existence of thermoplastic elastomers such as thermoplastic polyurethane has established a range of properties between two otherwise separate polymer categories, thermoplastics and elastomers. Thermoplastic polyurethanes exist as block copolymers of thermoplastic and elastomeric segments, blending the properties of each component and reducing or eliminating crosslinking between polymer chains^{146,171}. This is in contrast to thermosetting elastomers which contain extensive crosslinking and are not readily soluble or “reversible” in their processing^{146,172}. For this reason, electrospinning of polyurethanes with little to no thermoplastic components has not been explored to an appreciable degree and would not be trivial to achieve in the BVM lab with the current electrospinning protocol.

Natural Rubber (NR): Natural rubber is also a polymer made primarily of polyisoprene and thus also an elastomer¹⁷³. It has been used in medical applications like condoms and gloves as well as biomembranes for angiogenesis and bone regeneration¹⁷⁴. However, because of the crosslinking inherent to its structure NR is difficult to electrospin, and the only electrospinning literature referencing natural rubber used the polymer as an additive in electrospun polymer blends. This included additions up to 50% in PCL scaffolds, resulting in a decrease in fiber diameter from 1368 to 210 μm and concentrations of 0-50% of epoxidized NR in PLA scaffolds, also reducing fiber diameter^{175,176}. Neither study reported values for Young's modulus and did not comment on scaffold flexibility.

Of the materials selected using CES for further investigation, tPUs, collagen, and PCL were found to have substantial prior evidence of electrospinning and tissue engineering success with no other apparent limitations that would hinder their ability to perform properly in the setting of the BVM lab. The final section of the flexible polymer materials selection process involved comparing the costs of polymers and the solvents required to dissolve them.

6.2.1.3 Material Cost

While one of the original reasons for moving to an in-house scaffold fabrication technique from purchased ePTFE tubing was because of the high cost of the latter, PLGA is quite expensive as well. At approximately \$54 per gram, using PLGA in experiments that require several replicates or are testing several factors is quite costly, and limits the

ability to establish confidence in results from experimentation. Thus, the cost of the remaining flexible polymers and their proposed solvent(s) was tabulated and compared as a final check for feasibility (Table XXVII and XXVIII).

Table 27. Costs of Flexible Polymers for Electrospinning

Material	Mass Range (g)	Price per gram (\$/g)	Source
Thermoplastic Polyurethane	>10,000*	0.0019*	Lubrizol
Collagen	0.005 - 1	120.00 – 10,350.00	Sigma Aldrich
Polycaprolactone	5 - 500	8.58 – 0.39	Sigma Aldrich

*Material was quoted in ranges beginning at 50 lbs and increasing to 2,400 lbs. samples of 1 lb were donated for testing purposes.

Table 28. Costs of Proposed Solvents for Flexible Polymer Electrospinning

Solvent	Volume Range (mL)	Price per mL (\$/ml)	Source
Acetone	1000 – 16,000	0.032 – 0.014	VWR
Chloroform	100 – 2,500	0.364 – 0.053	Acros Organics
Methylene Chloride	100 – 6,000	0.383 – 0.057	Acros Organics
Dimethylformamide (DMF)	100 – 6,000	0.507 – 0.0431	Acros Organics
1,1,1,3,3,3,-hexafluoro-2-propanol (HFIP)	0.01 – 0.25	6,925 – 2,843	Acros Organics

All polymers and solvents were sourced from vendors mentioned directly in literature or from those with which the BVM lab has worked with in the past. Solvents were chosen based on their appearance in electrospinning literature with the PCL, collagen, and tPU.

As provided in Table XXVII, the cost of collagen per gram is significantly higher than

that of PCL or tPU. The average cost was even higher than that of PLGA and would provide the same prohibitory effects on high-volume experimentation that PLGA has caused in the past. Additionally, acetone, chloroform, methylene chloride, and DMF were all relatively inexpensive when compared to PLGA; However, HFIP, a commonly used solvent in published literature for a wide variety of electrospinning applications, is several times more expensive than chloroform; As such, the use of HFIP certainly would also inhibit the ability of the lab to do thorough research. Additionally, HFIP is a solvent used to dissolve polyoxymethylene (POM or Delrin), the material from which the electrospinning collector is fabricated. For these reasons, PCL and tPU were selected to be spun with acetone, chloroform, and methylene chloride. These solvents were shown to dissolve PCL and tPU in previous electrospinning literature and were readily available in the BVM lab. DMF was not chosen for this set of experiments to reduce the total number of scaffolds spun, however its future use as an electrospinning solvent in the BVM lab was recommended.

Based on the results of the materials selection process, PCL and tPU were the polymers deemed most appropriate for the requirements of the BVM lab based on material properties, information regarding their electrospinnability and compatibility with tissue engineering applications, and their cost. As noted previously, PCL and tPUs have been electrospun for use in tissue engineering applications from several sources. They all achieved favorable fiber diameter results, publishing values that are comparable or better to those typically achieved using PLGA in the BVM lab. The methods, equipment, solvents, and parameters used were also highly compatible with the capabilities of the

BVM lab. Additionally, any published cell response data suggested that both materials were suitable for use as a scaffold for short-term tissue engineering constructs. A more comprehensive summary of relevant electrospinning and tissue engineering literature regarding the two polymers will be presented herein:

6.2.1.4 Polycaprolactone

Polycaprolactone (PCL) is a synthetic biodegradable polymer that has been researched and used extensively in the field of tissue engineering and has been a component of several FDA-approved medical devices, such the Neurolac nerve guide, the Nasopore ear wick, and Osteopore PCL scaffold bone void filler^{177–182}. PCL has a glass transition temperature of -60 °C and thus is always in a rubbery, flexible state in applications as a biomaterial, making it an attractive option for use with matching the mechanical properties of flexible tissues. Additionally, PCL naturally degrades in the body through hydrolysis and enzymatic action, taking up to 1 year to fully decompose^{178,183}. It is commonly blended with several other polymers including PLA to alter its mechanical and degradation properties for a particular application¹⁴⁵. PCL has also been used for electrospinning research, specifically for the purposes of tissue engineering scaffold fabrication. A few specific examples of electrospun PCL for tissue engineering will be highlighted below:

Bosworth et al. attempted reduce the use of more harmful solvents in polymer electrospinning by characterizing the effect of several processing parameters on a

solution of PCL and acetone mixed at 5, 7.5, and 10 %wt/v, including changes in voltage, gap distance, and flow rate. A wide range of microscopic morphologies were formed including amorphous, nonfibrous polymer mats, randomly arranged cylindrical fibers, and severely beaded fibers. The smallest non-beaded fiber diameter achieved was approximately 200 nm, which steadily increased on average as PCL concentration increased up to ~3000 nm¹⁶⁴. These results suggest fibers much smaller than those achieved with PLGA may be possible in the BVM lab when using PCL, and provided a specific set of parameters to be attempted.

Lee et al. sought to characterize other solvents for PCL electrospinning, specifically methylene chloride (MC), mixtures of MC and *N,N*-dimethylformamide (DMF), and MC-toluene mixtures at various PCL concentrations between 10 and 15wt%. MC, toluene, and DMF are good, poor, and nonsolvents for PCL respectively, and have medium, low, and high dielectric constant values. Once again, a wide range of fiber morphology was achieved, including amorphous polymer mats, cylindrical fibers, and large beads along relatively small fibers. These fibers ranged in size between 200 and 5500 nm, with the smallest unbeaded fibers forming in 40:60 MC:DMF solutions. The addition of DMF caused a drastic decrease in fiber diameter from 5500 nm for pure MC solutions due to a decrease in surface tension and viscosity and an increase in conductivity and dielectric constant³⁷. This means that the force acting against the formation of a jet and the thinning of a fiber were lessened and the strength of the electrical conduction between the solution and mandrel were increased, leading to smaller fibers. This served as additional support that PCL was a reasonable choice for polymer

electrospinning, and provided insight into important trends experienced with electrospinning parameters when using PCL.

Wu et al. attempted to mimic the circumferential orientation of collagen fibrils in the native blood vessel media and axially oriented intima fibers to facilitate proper endothelialization and cell orientation through electrospinning of PCL. PCL was dissolved in chloroform at 10wt.% with an addition of dimethylformamide (additional 10wt% of original solution) to adjust the evaporation weight and surface tension of the solution. Fiber diameter ranges of 300-500 nm were achieved, and seeding these scaffolds with bovine aortic endothelial cells (BAECs) resulted in observable cell attachment and alignment¹⁶⁵. This suggests that electrospun PCL can provide an appropriate substrate for cell adhesion, and specifically for endothelial cells.

Yoshimoto et al. characterized an electrospun PCL construct for the use in bone tissue engineering. PCL dissolved in chloroform was spun into a fibrous mat with a mean fiber diameter of 400 ± 200 nm (all fibers between 20-5000 nm), sterilized with 70% ethanol, conditioned with collagen solution, and then seeded with mesenchymal stem cells harvested from the bone marrow of 3 to 7-day-old neonatal Lewis rats. It was observed that cells adhered to the porous PCL structure, differentiated as expected, and produced an extracellular matrix of collagen throughout the synthetic scaffold¹⁶⁶. It was also noted that unlike previous trials performed with PLGA scaffolds, the PCL scaffolds did not exhibit any shrinkage during sterilization, cell seeding, or beyond. Yoshimoto's

work was especially helpful because it showed that PCL could be readily incorporated into the BVM lab protocol, even using the same solvent and sterilization technique, and would produce an efficacious tissue engineered construct.

Pektok et al. electrospun several 2mm internal diameter PCL tubular structures to compare against the performance of ePTFE as scaffolds for vascular grafts in rats. PCL was dissolved in a 7:3 mixture of chloroform and ethanol at 15% (wt./vol.), and the resulting electrospun scaffolds had mean fiber diameter values of 1.90 μm . 1 cm lengths of scaffold were implanted between the renal arteries and the aortoiliac bifurcation in 30 rats, 15 for each material. The PCL scaffold group had higher endothelial cell coverage in less time, higher cell infiltration, observable neoangiogenesis, and no stenotic lesions, all improvements over the ePTFE group²⁰. However, there was a noticeable increase in chondroid metaplasia and calcification, likely due to a combination of local immune responses and local pH decrease due to PCL degradation. Still, the impressive cell coverage is reason to believe that PCL may be an acceptable scaffold material for the purposes of the BVM lab.

PCL was initially considered for testing in the BVM lab due to its low Young's modulus and ultimately selected for future experimentation based on a wealth of prior knowledge developed from various electrospinning and tissue engineering research efforts. Overall, literature showed that PCL can achieve a wide range of fiber diameters, a significant portion of which are equivalent to or below those currently achieved in the

BVM lab with PLGA. Additionally, PCL has been used in several tissue engineering and applications, specifically as an electrospun construct on which cells have been cultured. In this way, prior research has shown that the techniques used for BVM setups should translate well from PLGA to PCL.

6.2.1.5 Thermoplastic Polyurethane

Thermoplastic polyurethane is a type thermoplastic elastomer comprised of alternating blocks of hard and soft monomer groups¹⁴⁶. The relative amounts of each segment, and the composition of the soft segments can be tailored to achieve a wide range of mechanical and biostability properties¹⁴⁵. tPUs have been used in long-term implantable devices such as the coating of pacemaker leads, and can also be manufactured as a bioabsorbable, taking advantage of the quicker degradation properties of soft segments made of PLA, PGA, PCL, or PEO¹⁴⁵. Several types of thermoplastic polyurethanes have been used in electrospinning research, and specifically for electrospun scaffolds for tissue engineered constructs. A few example cases will be covered below:

Like Lee et al., Kidoaki et al. attempted to characterize the effect of various solvents and solvent mixtures on the result of polymer electrospinning, this time with polyurethane. Concentrations of 10 to 17.5 wt.% were used with solvent mixtures of tetrahydrofuran and DMF of varying composition. It was observed that fiber diameter decreased as concentration, gap distance, and flow rate all decreased for a given solvent

mixture. Additionally, fiber diameter appeared to decrease and the density of fiber packing appeared to increase with increasing DMF content¹⁴⁷. This suggested that tPU scaffold morphology and fiber diameter can be altered by methods previously explored in the BVM lab.

Similar to work done by Pektok et al., Bergmeister et al. investigated the use of an electrospun synthetic polymer as a possible option for a small-diameter vascular graft, as previously tested synthetic materials have suffered from inherent surface thrombogenicity and intimal hyperplasia development compared to native tissue. Electrospun thermoplastic polyurethane scaffolds with mean fiber diameter of 880 nm were sterilized in 4°C PBS and then implanted at the aortic interposition of 40 Sprague-Dawley rats. Upon retrieval and analysis it was observed that 95% of prostheses were patent with no instances of noticeable degradation and no evidence of foreign body response¹⁸. Furthermore, it was noted that based on the time points at which the scaffolds were removed that cell ingrowth, cell differentiation, and collagen ECM formation occurred quickly and suggest that electrospun polyurethane may be a viable option for synthetic small-diameter vascular grafts. This showed that some native cell response is achievable for small-diameter vessel constructs with tPU scaffolds.

Like Bergmeister et al., Grasl et al. also used electrospun polyurethane to form vascular grafts, specifically to characterize the mechanical behavior and endothelial cell adhesion. Scaffolds were spun with a thermoplastic polyurethane dissolved in 1,1,1,3,3,3-

hexafluoro-2-propanol onto a rotating and translating cylindrical mandrel. Scaffolds were sterilized with UV irradiation, seeded with HUVECs, and left to culture in static conditions within a humidified incubator. Constructs were evaluated based on the presence of or absence of EC adhesion markers E-selectin, ICAM-1, and VCAM-1, which can be used as an indication of inflammatory response activation. While previously tested vascular grafts made of materials like Dacron have stimulated this response and resulted in higher rates of thrombosis, these markers were present in much lower levels in the tPU grafts tested. Through mechanical testing it was also observed that the tensile strength of tPU grafts exceeded that of natural blood vessels based on data from rat aorta tests¹⁴⁸. These results suggest that tPU scaffolds can not only foster cell adhesion and remain intact as a vascular graft, but that they may provide a noticeable improvement compared to other synthetic scaffold materials.

From these examples, it's clear that PCL and tPUs have many of the necessary and desired properties of a material for electrospinning in the BVM lab. For these reasons, PCL and tPUs were investigated further within the context of the electrospinning setup in the BVM lab to compare with typical PLGA results and limitations.

6.2.2 Electrospinning

6.2.2.1 Preliminary Trials

Based on the materials selection results, PCL and tPU were determined to be two of the most viable flexible polymers to replace PLGA in the BVM lab. PCL (Sigma

Aldrich, 440744) and two types of tPU (Lubrizol Lifesciences, PC3575A and PC3585A) were acquired and electrospun using the same basic protocol as BVM lab PLGA electrospinning. These PCL and tPU sources were chosen due to their similarity to those presented in literature involving electrospinning for tissue engineering applications. Certain parameters, however, were altered based on published literature results in an attempt to produce similar scaffolds. Scaffolds of these new materials were spun in several different trials, used to test a set of parameters, observe the results, and make informed decisions about the next round of scaffolds to be produced. All electrospinning was done in accordance with the updated BVM lab electrospinning protocol, found in Appendix H. The full description of materials, spins, and their respective parameters for the preliminary trial are listed below (Table XXIX).

Table 29. As-Planned Preliminary Trial Parameters for Flexible Polymer Electrospinning

Spin Number	Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
1P	PCL	Acetone	8.66	3	-12	10
2P	PCL	Acetone	15	5.5	-12	10
3P	PCL	Chloroform	10	6	-13	10
4P	PCL	Methylene Chloride	12	5.5	-12	10
5P	PC-3575A	Chloroform	15	5.5	-12	10
6P	PC-3575A	Chloroform	7.5	5.5	-12	10
7P	PC-3585A	Chloroform	15	5.5	-12	10
8P	PC-3585A	Chloroform	7.5	5.5	-12	10

As a note, all fiber diameter and pore area measurements presented were taken on images of 600x magnification during this preliminary study unless stated otherwise. Attempts were made to measure the proximal, middle, and distal portions of each scaffold, however this was not always possible if scaffolds could not be successfully

removed from their mandrels, as was commonly the case with tPU scaffolds, or if large sections of the scaffold were used for other experimentation.

Results and Observations

Spin 1P and 2P

Purpose:

The parameter set used for Spin 1 was influenced by the work of Bosworth et al., who used 8.66wt% PCL in acetone spun at 3 ml/hr¹⁶⁴. Voltage and gap distance parameters were not specified so standard BVM electrospinning protocol values were used. Spin 2P was intended as a control using standard parameter values for all aspects of the process to compare against Spin 1P.

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PCL	Acetone	8.66	3	-12	10
PCL	Acetone	15	5.5	-12	10

Observations:

Unfortunately, PCL did not fully dissolve in acetone and thus no electrospinning could be done (Figure 81).

Figure 81. PCL pellet agglomerate after exposure to acetone.

Spin 3P

Purpose:

Spin 3P was another PCL spin based on parameters described by Yoshimoto et al. and was chosen due to its use of chloroform and general simplicity¹⁶⁶. Because of the use of chloroform and nearly-identical electrospinning parameters as those found in the PLGA, Spin 3P acted as a direct comparison between PLGA and PCL.

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PCL	Chloroform	10	6	-13	10

Observations:

There were complications with the electrospinning setup during the fabrication of Spin 3P: the distal end of the mandrel normally is held in place by friction against a

freely rotating spring-loaded component. The spring was not engaged properly and the rotation of the mandrel caused rubbing against the spring-loaded component, generating heat and melting the distal portion of the scaffold (Figure 82).

Figure 82. Spin 3P scaffold (top scaffold) with partially melted then solidified distal end (left). Spin 4 is below as a reference.

The scaffold was also difficult to remove from the mandrel, particularly near the distal end, possibly because of the unintended heating during electrospinning. SEM images of the scaffold are included below along with average fiber diameter and pore area values (Figure 83, Table XXX).

Figure 83. SEM images of Spin 3P at 60x (left) and 600x (right) at proximal (top), middle (middle), and distal (bottom) positions along the scaffold.

Table 30. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 3P

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	10.68	3.35	717.12	1011.67
Middle	8.93	1.07	775.99	1279.88
Distal	12.83	4.30	429.75	826.74
Average	10.813	2.91	585.46	1015.31

Spin 4P

Purpose:

The use of methylene chloride and the general parameter set for Spin 4P was inspired by Lee et al. who observed average fiber diameter values of approximately 5500 nm (5.5 μm) at with similar processing conditions. Despite the fact that the reported fiber diameters are not particularly impressive in the context of results achieved in the BVM lab with PLGA, MC has a noticeably lower viscosity compared to chloroform (0.449 and 0.563 mPa*s, respectively), and was tested to investigate the potential benefit it could provide compared to chloroform¹⁰⁷.

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PCL	Methylene Chloride	12	5.5	-12	10

Observations:

During electrospinning, several observations were made: The solution was noted to be much more viscous than previous PCL and PLGA solutions during uptake into the syringe and there was only 2 mL of solution in the vial despite using 4.32 mL of MC and 0.7826 g of PCL, indicating a significant reduction of volume due to mixing.

Additionally, a stable jet did not form at -12 kV so the voltage was increased to -16 kV, the point at which dripping ceased and a constant jet formed. Finally, PCL fibers preferentially built up on the middle of the mandrel, creating a scaffold of variable wall thickness (Figure 84). This effect was more evident during electrospinning and the gradient in thickness decreased over the duration of the spin, however it was still noticeable after electrospinning had concluded.

Figure 84. Spin 4P with parallel lines superimposed to show the scaffold tapering in thickness.

SEM images and subsequent fiber and pore measurements were taken of the proximal and distal portions of the scaffold; the middle portion of the scaffold was reserved for further testing (Figure 85, Table XXXI).

Figure 85. SEM images of Spin 4P at 60x (left) and 600x (right) at proximal (top) and distal (bottom) positions along the scaffold.

Table 31. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 4P

Location	Fiber Diameter (μ m)		Pore Area (μ m ²)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	8.04	5.27	205.95	304.39
Distal	10.70	1.74	415.65	573.57
Average	9.37	3.50	262.93	403.44

Spin 5P

Purpose:

Processing parameters in literature for thermoplastic polyurethane electrospinning varied significantly, and so preliminary trials with the 2 tPUs available were simply done to find an acceptable polymer concentration on which to base future electrospinning trials. Spins 5P is the high polymer concentration trial for PC-3575A or simply “75A”. Standard BVM electrospinning parameters were used as a baseline on which to base all future modifications.

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PC-3575A	Chloroform	15	5.5	-12	10

Observations:

During the electrospinning process the flow rate was lowered to 4.0 ml/hr and applied voltage was increased to -18kV to prevent dripping and to maintain a steady polymer jet. Removing the scaffold from the mandrel was noticeably more difficult than with PLGA or PCL scaffolds. Samples sized for SEM imaging were cut directly off the mandrel, while several lab members performed various manual removal techniques and even assisted their efforts with dips in 70% ethanol, however removal of an intact scaffold was ultimately not successful (Figure 86 and 87, Table XXXII).

Figure 86. Spin 5P scaffold during removal from the mandrel. Peeling instead of smooth sliding made removal of a fully intact tubular scaffold impossible.

Figure 87. SEM images of Spin 5P at 60x (left) and 600x (right) at proximal (top), middle (middle), and distal (bottom) positions along the scaffold.

Table 32. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 5P

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	9.49	4.71	313.10	392.11

Spin 6P

Purpose:

Spin 6P was the low polymer concentration group for tPU 75A, and was set to spin at standard BVM lab electrospinning parameters. 7.5wt% was chosen as a semi-arbitrary value lower bound for polymer concentration for preliminary testing.

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PC-3575A	Chloroform	7.5	5.5	-12	10

Observations:

Although standard BVM electrospinning parameters were used to begin the spin, the flow rate was quickly decreased to 4 mL/hr and then to 2 mL/hr and voltage increased to -15kV in an effort to reduce dripping. Scaffold removal experienced issues very similar to those encountered with Spin 5P, and only a portion of the proximal end was salvaged for SEM imaging (Figure 88, Table XXXVIII.).

Figure 88. SEM images of Spin 6P at 60x (left) and 600x (right) at the proximal position along the scaffold.

Table 33. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 6P

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	13.28	4.99	383.71	554.47

Spin 7P

Purpose:

Spin 7P served the same purpose as 5P for the 85A tPU material: acting as an equivalent to PLGA for standard BVM electrospinning parameters and providing results for a relatively high polymer concentration spin.

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PC-3585A	Chloroform	15	5.5	-12	10

Observations:

Spin 7P was mixed as the high polymer concentration 85A tPU sample, however the solution was noticeably more difficult to uptake into the syringe than any previous sample due to high viscosity. Additionally, the solution appeared resistant to any amount of electrostatic force applied via the power supply, and ultimately no jet formed and no scaffold could be spun.

Spin 8P

Purpose:

Spin 8P was the low polymer concentration sample for 85A, and used the same concentration value of 7.5wt% as Spin 6P to establish a direct comparison between their results.

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PC-3585A	Chloroform	7.5	5.5	-12	10

Observations:

Similar to the low concentration spin for 75A tPU material, spin 6P, the parameters defined in the BVM electrospinning protocol were altered to achieve optimal spinning conditions: Voltage was increased to -13kV and flow rate was incrementally decreased to 2.0 mL/hr to maintain a stable jet and mitigate dripping. Similar to the two previous tPU scaffolds 5P and 6P, the removal of Spin 8P from the mandrel was difficult

and only resulted in 1 usable sample for SEM imaging. Spin 8P did not appear to form a fibrous structure, rather it appeared as amorphous areas of solidified polymer with trails of fiber-like shapes occasionally sprouting from these areas, and thus a fiber diameter measurement could not be taken (Figure 89, Table XXXIV).

Figure 89. SEM images of Spin 8P at 60x (left) and 600x (right) at the proximal position along the scaffold.

Table 34. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 8P

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	-	-	651.62	1605.81

Fiber diameter and pore area measurements from each of the preliminary trial scaffolds were then compiled and tabulated to perform a direct, statistical comparison (Table XXXV).

Table 35. Summary of Preliminary Flexible Material Study Fiber Diameter and Pore Area Results

Scaffold	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
3P	10.813	2.91	585.46	1015.31
4P	9.37	3.50	262.93	403.44
5P	9.49	4.71	313.10	392.11
6P	13.28	4.99	383.71	554.47
8P	-	-	651.62	1605.81

It was determined that fiber diameter results only differed significantly between Spins 4P and 6P through use of a general linear model with Tukey pairwise comparisons (Figure 90).

Figure 90. Box and whisker plot of preliminary flexible polymer scaffold fiber diameter results. *, $p < 0.05$ between groups.

Additionally, it was determined that scaffold 3P had significantly higher average pore area results than all but scaffold 8P (Figure 91).

Figure 91. Box and whisker plot of preliminary flexible polymer scaffold pore area results.

*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ between groups.

In addition to these quantitative fiber diameter and pore area results, qualitative observations were made when handling all flexible polymer scaffolds. PCL and both tPUs were significantly more flexible and elastic than PLGA scaffolds; both had the ability to fold over 180° without signs of plastic deformation.

These results served as a starting point from which to base another round of flexible polymer electrospinning in an attempt to optimize fiber diameter and pore size to reach equivalence or superiority to the current typical PLGA scaffold produced in the BVM lab.

6.2.2.2 Second Flexible Polymer Trials

Based on the results of the flexible polymer electrospinning trial with PCL and tPUs, several protocol changes were made to either optimize fiber and pore size or to induce the formation of a fibrous structure when one did not form previously.

Specifically, acetone as a solvent for PCL was eliminated and polymer concentration was lowered for all materials. Several sources cite this as a method by which fiber diameter can be reduced in electrospun scaffolds^{107,126,128,129}. Additionally, the tPU manufacturer recommended polymer solutions between 2 and 7 wt.%. The applied voltage was still set to -12kV at the beginning of each electrospinning session, however it was adjusted as needed throughout each spin (Table XXXVI).

Table 36. Follow-up Trial Parameters for Flexible Polymer Electrospinning

Spin Number	Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
1F	PCL	Chloroform	7.5	5.5	-12	10
2F	PCL	Methylene Chloride	7.5	5.5	-12	10
3F	PC-3575A	Chloroform	5	5.5	-12	10
4F	PC-3585A	Chloroform	5	5.5	-12	10

Results and Observations

Spin 1F

Purpose:

The reduction in polymer concentration in Spin 1F of the follow-up flexible polymer trials was done to reduce fiber diameter and pore size, a trend that has been

observed several times in other electrospinning efforts^{96,164}. All other parameters were held constant with those outlined in the standard BVM lab protocol

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PCL	Chloroform	7.5	5.5	-12	10

Observations:

Although all parameters aside from polymer concentration were held constant from the BVM electrospinning protocol, voltage was increased to -15kV for the majority of the Spin 1F trial to facilitate the formation of a proper polymer jet. Additionally, the deposition of PLGA solution on the mandrel was heavily skewed towards the proximal end, with little to no coverage on the distal end. The syringe pump was angled slightly towards the distal end midway through the spinning process to encourage even deposition, however the produced scaffold was still noticeably lesser in length compared to standard spins, and the distal coverage appeared to be merely superficial.

From observation of SEM images of scaffold 1F, it was immediately obvious that this trial did not form a fibrous scaffold but rather larger areas of amorphous polymer deposition that was significantly more porous on the proximal end than the distal, which eliminated the possibility of taking fiber diameter measurements from this sample, and pore area measurements from the distal end (Figure 92, Table XXXVII).

Figure 92. SEM images of Spin 1F at 60x (left) and 600x (right) at proximal (top) and distal (bottom) positions along the scaffold. The features visible on the polymer areas in the 600x images are indicative of the surface finish on the mandrel used for electrospinning.

Table 37. Fiber Diameter and Pore Area of Second Flexible Material Study, Spin 1F

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	-	-	962.57	949.66
Distal	-	-	-	-

Spin 2F

Purpose:

Spin 2F was conducted with a similar reduction in polymer concentration as an attempt to decrease average fiber diameter and potentially establish a lower bound for this polymer-solvent combination and its ability to produce desirable fibers.

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PCL	Dichloromethane	7.5	5.5	-12	10

Observations:

A similar disparity in coverage compared to Spin 1F was noted for the first several minutes of the spin, however this corrected over time. Additionally, the flow rate was decreased to 4.0 mL/hr and voltage increased to -15kV to limit dripping and maintain a constant polymer jet.

Contrary to the results of Spin 1F, Spin 2F produced SEM images that showed distinct fiber formation with an average fiber diameter of 3.89 μm . This is noticeably lower than that Spin 4P, the previous PCL-MC spin with a 15wt% polymer concentration. The appearance and distribution patterns of the fibers was somewhat different than those produced using PLGA, however the fibrous, porous nature of the scaffold is quite clear (Figure 93, Table XXXVIII).

Figure 93. SEM images of Spin 2F at 60x (left) and 600x (right) at proximal (top) and distal (bottom) positions along the scaffold.

Table 38. Fiber Diameter and Pore Area of Second Flexible Material Study, Spin 2F

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	4.41	4.01	220.53	444.24
Distal	3.41	1.46	211.56	302.62
Average	3.89	2.96	216.36	383.86

Spin 3F

Purpose:

Spin 3F was an attempt to fabricate a tPU scaffold with a reduced average fiber diameter compared to Spin 6P, which used a concentration of 7.5wt% in CHCl₃.

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PC-3575A	Chloroform	5	5.5	-12	10

Observations:

As with many of the flexible polymer spins, flow rate was decreased to 5.0 mL/hr and voltage increased to -19kV. Additionally, while the appearance of the polymer jet drawing from the needle tip may change slightly from spin to spin, the jet is usually visible for several inches or more of the gap distance. In the case of Spin 3F it was only visible for approximately 1 cm before it separated into a more dispersed, less visible spray.

SEM images showed that Spin 3F did not form fibers at all, but an amorphous porous structure more similar to the results of Spins 8P and 1F. As was the case with those spins, fiber diameter measurements could not be taken from the images. While the scaffold was not fibrous in nature, it did appear to form a consistent, tubular structure. However,

similar to previous tPU spins, the scaffold tended to rip, bunch up, and roll over itself, making scaffold removal impossible (Figure 94 and 95, Table XXXIX).

Figure 94. Spin 3F scaffold removal attempts, showing tearing and rolling (top), bunching and rolling (middle), and scraps cut from the mandrel after all methods had been exhausted.

Figure 95. SEM images of Spin 3F at 60x (left) and 600x (right) at the proximal position along the scaffold.

Table 39. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 3F

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	-	-	1092.59	1691.53

Spin 4F

Purpose:

The mixing and electrospinning of Spin 4F was done identically to the procedure of Spin 3F, save for the use of tPU 85A instead of 75A. These methods and parameters were chosen with the hope of forming a more fibrous structure compared to Spin 8P.

Parameters:

Material	Solvent	Concentration (wt.%)	Flow Rate (ml/hr)	Voltage (kV)	Gap Distance (in)
PC-3585A	Chloroform	5	5.5	-12	10

Observations:

Similar decreases in flow rate and increases in applied voltage were also performed as needed. Unlike Spin 3F, however, the needle tip became clogged with solidifying polymer solution several times throughout the duration of the spin. To rectify this the voltage source and syringe pump were switched off, the tip was cleared manually, and the process was resumed.

The similarities between Spins 3F and 4F remained present when SEM images were observed. Instead of a fibrous structure, a network of amorphous, porous polymer appeared to have been deposited during electrospinning (Figure 96, Table XL).

Figure 96. SEM images of Spin 4F at 60x (left) and 600x (right) at the proximal position along the scaffold.

Table 40. Fiber Diameter and Pore Area of Preliminary Flexible Material Study, Spin 4F

Location	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
Proximal	-	-	735.21	1089.85

Pore area results from the secondary flexible polymer trial scaffolds were similarly compiled and tabulated for direct comparison (Table XLI). Because only 1 scaffold successfully formed a fibrous structure from this round of electrospinning trials, the results of it were compared against those from the preliminary flexible polymer trial (Table XLII).

Table 41. Summary of Secondary Flexible Material Study Fiber Diameter and Pore Area Results

Scaffold	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
1F	-	-	962.57	949.66
2F	3.89	2.96	216.36	383.86
3F	-	-	1092.59	1691.53
4F	-	-	735.21	1089.85

Table 42. Summary of Successful Flexible Material Study Fiber Diameter and Pore Area Results

Scaffold	Fiber Diameter (μm)		Pore Area (μm^2)	
	Mean	Std. Dev.	Mean	Std. Dev.
3P	10.813	2.91	585.46	1015.31
4P	9.37	3.50	262.93	403.44
5P	9.49	4.71	313.10	392.11
6P	13.28	4.99	383.71	554.47
2F	3.89	2.96	216.36	383.86

It was determined that the average fiber diameter of 2F was significantly lower than those of any other successfully electrospun flexible polymer parameter set (Figure 97).

Figure 97. Combined fiber diameter results from the preliminary and secondary flexible polymer trials. *, $p < 0.05$ between groups. #, $p < 0.001$ between all other groups.

It was also determined that a significant difference in pore area existed between all scaffolds spun in the second flexible electrospinning study save for Spins 3F and 4F (Figure 98).

Figure 98. Pore area results for the secondary flexible polymer trials. *, $p < 0.05$ between groups. #, $p < 0.001$ between all other groups.

These results suggested that a PCL scaffold can be spun to have similar fiber diameter and pore area compared to typical PLGA scaffolds spun in the BVM lab. When handling the scaffolds, it was also immediately apparent that PCL scaffolds were much more flexible than standard PLGA scaffolds spun in the BVM lab. Based on the results of the materials selection process, PCL and tPU appeared to be the only polymers that had been shown to perform as an effective cell scaffold in a tissue engineering setting and that would fit the needs of the BVM lab. The electrospinning results largely eliminated the use of tPU due to the lack of consistent, fibrous scaffolds that could be easily fabricated with the current BVM electrospinning setup, and the inability to remove tPU scaffolds from their mandrels in a consistent, acceptable manner. This left PCL as the only viable flexible polymer option for more complex BVM setups.

Next the kink radius of each type of scaffold was determined for both materials to evaluate the usefulness of increased flexibility of the PCL scaffold. This provided a more accurate, applicable test for flexibility rather than using accepted elastic modulus values, and is explored in greater detail below.

6.2.3 Characterizing Scaffold Flexibility

While the flexibility of electrospun polymer scaffolds may be gleaned from literature sources that state the “true” value of a particular mechanical property, tests performed in the BVM lab and in literature have presented results significantly different

than those values displayed in CES, for example^{141,162}. The fibrous, porous structure of an electrospun scaffold does not behave in the same manner as a solid section of the same material and bulk geometry, and any alignment or directionality in the fibers will also introduce an element of anisotropy to the scaffold.

Because of this difference a direct measurement of scaffold flexibility was tested by determining the maximum kink radius of electrospun scaffolds of each material. Using ISO 7198:1998 – Tubular Vascular Prostheses, scaffolds of ePTFE, PLGA, and PCL were curved around cylindrical templates of incrementally decreasing radii to determine the radius at which kinking occurred. A kink radius test was used because it characterized the electrospun scaffolds in their usable form, and tested them in bending, which is the geometry experienced in more tortuous BVM bioreactor designs. Because they could not be removed from the mandrel in a single, tubular piece after electrospinning, tPU scaffolds were not used for this test and were considered unsuitable for BVM lab needs.

The cylindrical templates were laser cut from 0.242 in. thick medium density fiberboard (MDF) using a CO₂ laser cutting machine (Universal Laser Systems, X2-660) provided by the Cal Poly College of Engineering Machine Shops. The templates were engraved with their respective diameter values, and ranged between 13 to 100 mm in diameter in increments of 3 mm, as per ISO 7198:1998 (Figure 99) (Appendix J).

Figure 99. ISO 7198:1998 kink radius templates.

6.2.3.1 ePTFE

ePTFE tubing of 3.5 mm inner diameter was used as a reference material for flexibility testing; It was already determined that ePTFE tubing could be conformed into more complex shapes for more tortuous device testing according to results in the thesis of Dalton Chavez⁸. After wrapping the tubing around all circle templates in order of decreasing radius it was determined that the ePTFE did not kink at any diameter (Figure 100).

Figure 100. Representative images of ePTFE tubing kink testing. ePTFE did not kink at any radius, including with the smallest circle templated of 7.5 mm radius.

6.2.3.2 PLGA

The same kink radius-testing procedure was used on scaffolds of PLGA spun using the newly revised BVM lab electrospinning protocol (Appendix H). The PLGA unexpectedly conformed to the size of nearly all templates before kinking at the 25 mm diameter/12.5 mm radius templates. However, bending the PLGA scaffold around each template required much more force than ePTFE, and it was observed that the scaffold was permanently deformed to some degree after every test (Figure 101 and 102).

Figure 101. Representative images of PLGA scaffold kink testing. Images on the left show the scaffold during testing, while images on the right indicate the permanent deformation observed after the respective template.

Figure 102. PLGA tink testing images with the 25 mm diameter template (top) with which kinking first occurred, and with the 13 mm diameter template (bottom).

6.2.3.3 PCL

Finally, a PCL scaffold was also subjected to the same kink radius-testing experiment (Appendix J). Interestingly, the scaffold kinked immediately upon application of light bending force, and displayed noticeable kinking when tested on all circle templates (Figure 103). It was also observed that the material was much easier to deform than PLGA, and that most or all of the deformation was elastic in nature with a slight delay when recovering from the deflection (Figure 104).

Figure 103. Representative images of PCL scaffold kink testing. Kinking occurred immediately with all templates.

Figure 104. Manual flexion of an electrospun PCL scaffold. The scaffold was flexible and could be bent easily by hand (left), but experienced somewhat of a hysteresis effect when returning to its as-fabricated shape (right).

The handling of each scaffold material during kink testing supported the assumption that a PCL scaffold was noticeably more flexible than one of PLGA, however it also experienced kinking at much larger template radii. For this reason, the PCL scaffolds spun in these experiments did not appear to be suitable for the tortuous bioreactor designs published by Dalton Chavez⁸. Additionally, although the PLGA scaffold had a relatively small kink radius of 12.5 mm (25 mm diameter template), it was observed that bending the scaffold required a significantly larger force than either ePTFE or PCL, and that the deformation performed on the scaffold was permanent. This behavior was not like that of a blood vessel, and so it was reaffirmed that the current BVM lab PLGA scaffolds are not suitable for bending applications either. ePTFE tubing did not kink at any radius and was much more compliant than PLGA, and generally appeared to be the most suitable material for bending applications.

6.3 Discussion

The materials selection process for an alternative, flexible electrospinning material for use as a scaffold in the BVM lab was focused on 3 criteria: flexibility, as quantified by Young's modulus, previous literature evidence of electrospinning and tissue engineering use, and cost. Ultimately PCL and two very similar types of tPU were selected for comparison against PLGA for electrospinnability and scaffold kink radius. Ultimately, both tPUs could not be consistently fabricated into a fibrous, tubular scaffold that could be successfully removed from the mandrel while maintaining its shape. It was found that a parameter set existed with which PCL could be spun, and produced scaffolds with average fiber diameter values comparable to those achieved with the standard PLGA

electrospinning protocol. However, the kink testing results suggested that, while they required much less force to bend and flex than PLGA scaffolds, PCL scaffolds experienced kinking with any amount of bending, rendering them useless for more complicated, tortuous bioreactor setups. However, since fiber diameter values similar to those achieved with PLGA scaffolds were observed, it may be advisable to integrate the use of PCL scaffolds into straight vessel BVM setups. The flexibility of PCL scaffolds may be more accommodating to the handling required to fit a scaffold into the tight quarters of a BVM bioreactor, and the significantly lower cost will allow for more experimentation.

6.3.1 Materials Selection

The materials selection process began with a rudimentary comparison of several biomedical grade polymers within the Bioengineering version of the CES materials database software. Because materials of lesser stiffness/greater compliance than PLGA were desired, all materials that passed an imposed “biomedical use” limit were considered for the following literature review.

All materials with greater compliance than PLGA were reviewed for existing literature regarding their use as an electrospinning material. Furthermore, materials were considered based on their applications with intimate cell contact, particularly with electrospun structures of the material. This reduced a larger list of polymers down to just 3, PCL, tPU, and collagen.

The final criteria for flexible polymer materials selection was a cost estimate of each candidate material; both PLGA and its BVM lab predecessor ePTFE tubing are expensive and limit the capacity for experimentation within the lab, and so the costs of all material and proposed solvents were compared. The prohibitively high cost of collagen left only PCL and tPU as viable options for flexible polymer electrospinning, which was then performed to gain a basic understanding of their electrospinning characteristics and to attempt to simulate results previously achieved by PLGA solutions.

6.3.2 Electrospinning

PCL and two similar types of tPU were obtained and electrospun using a combination of electrospinning parameters presented in literature and those used in the typical BVM electrospinning protocol. While scaffolds spun from PCL were largely fibrous, porous structures with average fiber diameter results similar to that of PLGA, the same was not true for the tPU scaffolds. Most tPU scaffolds appeared as porous scaffolds made up of a matrix of droplets and/or beads, with little to no discernible fiber presence. Additionally, it was observed that the removal of tPU scaffolds from their mandrels was impossible to achieve successfully, and thus the tPU polymers were eliminated from consideration of a viable flexible electrospinning polymer.

6.3.3 Kink Testing

Finally, PCL and PLGA scaffolds were compared in terms of flexibility: Using a protocol outlined in ISO 7198 each scaffold was wrapped around circular templates of

decreasing size to observe the point at which each scaffold kinked, characterizing the maximum allowable bend radius in application. Surprisingly, PLGA did not show kinking until it was tested at a radius of 12.5 mm, while PCL showed obvious kinking at even the largest template, one of radius 50 mm. Such bending characteristics meant that the electrospun PCL scaffolds were not suitable for more tortuous bioreactor setups. However, it was also observed that the PLGA would retain some of the deformation that occurred during kink radius testing. Deflecting the PLGA scaffold required much larger forces than either PCL or ePTFE, to the point at which it was clear that electrospun scaffolds would also not properly replicate the mechanical properties of native blood vessels.

While information regarding the kinking behavior of electrospun polymer tubes is not widely known, there has been research into the kinking and buckling of more simplified tubes in the past: Using a simplified approach to the buckling behavior of thin-walled, circular cylinders it is asserted that the wall flexural stiffness is defined as such (Eq. 4):

$$D = \frac{Et^3}{12(1-\mu^2)} \quad (4)$$

In which D is the wall flexural stiffness, E is Young's modulus of the cylinder material, t is wall thickness, and μ is Poisson's ratio of the cylinder material¹⁸⁴. The D term is included in a larger equation that defines the axial compressive load required to cause buckling (Eq. 5):

$$N_x = k_x \frac{\pi^2 D}{l^2} \quad (5)$$

In which N_x is the axial load per unit width of circumference for a cylinder subjected to axial compression, k_x is a buckling coefficient subjected to axial compression, and l is the length of the cylinder¹⁸⁴. From this equation, wall flexural stiffness is positively correlated with the compressive load required for buckling, and thus E and t are positively correlated as well. While these findings were published specifically for structural members their general trends may be applied to relatively flexible polymer tubes as well.

This explanation is further supported by a review of the forces incurred within a tube during its bending. Bending a tube results in tri-axial material deformation along 3 orthogonal directions: normal to the cross section (axial), within the cross section (transverse), and through the wall thickness (radial) (Figure 105)¹⁸⁵.

Figure 105. Tri-axial deformation and strain experienced by a tube in bending¹⁸⁵.

As shown in the example of axial strain, a tube in bending experiences areas of tension and compression depending on their location relative to the neutral axis. The areas experiencing compressive force are those that exhibited kinking in the PLGA and PCL kink radius experiments. Given the fact that PLGA experienced kinking at much smaller radii than PCL and that PLGA has a higher Young's modulus value, it may be asserted that the positive correlation in compressive load to cause buckling and E shown in Equations 4 and 5 are also true for electrospun polymer tubes. It stands to reason that the trend in load with wall thickness may also exist when testing electrospun polymer tubes for a given material. While there will be practical limitations on the maximum wall thickness of a scaffold used for BVM purposes, it is advised that multiple PCL scaffolds with a variety of wall thickness values be spun and tested using the same kink radius test to observe any possible differences.

However, this does not account for the bending behavior of ePTFE; PTFE has a modulus value between that of PLGA and PCL, however the ePTFE tubing was observed to have similar compliance to PCL and had a comparable wall thickness to both scaffolds. It also exhibited the smallest kink radius, maintaining its tubular shape when tested on every template. This is due to the unique structure of ePTFE; the microscopic morphology of the tubular ePTFE grafts used in the BVFM lab consist of a network of fibrils connecting PTFE nodes, creating a porous, fibrous structure (Figure 106)^{186,187}. This nodular appearance of ePTFE may be replicated with an electrospinning material by

aligning the polymer fibers during fabrication in a radial manner, such that they form a spiraling set of “ribs” around the mandrel. Upon application of a compressive force to the scaffold, it is possible that the ribs compress and occupy the space between fibers to resist kinking, similar to ePTFE.

Figure 106. SEM images of the inside surface of ePTFE tubing from the BVM lab.

It was also noted during handling that the ePTFE tubing could be compressed axially with no apparent change in diameter, simply just contracting along its length, seemingly in violation of the Poisson effect¹⁸⁸. This is due to the compression of the fibrous areas of the ePTFE, filling the pores of the material without noticeably transverse strain. This is the same characteristic that allows for kink-less bending: the material between the neutral axis and the radius template experience a compressive force which compresses the fibril areas, moving the nodes closer together, effectively shortening that portion of the tube and preventing kinking (Figure 107). This effect has not manifested in any electrospun, fully fibrous structure fabricated in the BVM lab.

Figure 107. SEM images of ePTFE tubing experiencing contraction. The distance between nodes has decreased in areas of compression.

6.4 Conclusion

tPU and PCL were selected from a list of low-modulus, biocompatible materials and electrospun using several iterations of the BVM electrospinning protocol, and the kink radius of resulting PCL scaffolds were compared to electrospun PLGA scaffolds and ePTFE tubing. tPU scaffolds could not be successfully removed from their mandrels and PCL scaffolds performed significantly worse than PLGA and ePTFE in the kink radius test; because of this, neither material can be recommended for use in more tortuous BVM bioreactor designs. However, the electrospinning performance of PCL was similar to that of PLGA and offers handling and cost-related benefits if PCL is adopted as a standard BVM scaffold material. It is possible that kink resistance may be improved by increasing

either Young's modulus (either through morphological changes of PCL scaffolds or co-electrospinning with another polymer) or wall thickness, however this may reduce the flexibility of the scaffold as a whole, limiting its ability to conform to more tortuous pathways in a way similar to PLGA scaffolds. At this moment, ePTFE tubing is the most viable option for this particular BVM application, however tests of PCL scaffolds with significantly thicker walls is recommended.

However, it is possible that other flexible polymer options exist; The materials selection criteria were rather stringent regarding previous use of electrospun scaffolds in tissue engineering settings, however it may be the case that other flexible polymer options that have shown prior electrospinning results will also work favorably as a tissue engineering scaffold. Additionally, the list of flexible materials presented by CES was not exhaustive, and other options may be gleaned from outside literature sources. Finally, the kink radius test appeared to accurately characterize a relevant, applicable property of electrospun scaffolds. This test should be implemented with all future flexible polymer testing unless a more apt test is developed.

7. DISCUSSION AND CONCLUSION

7.1 Summary and Aims of this Thesis

Electrospinning is a polymer fabrication technique currently in use in the Cal Poly BVM lab as a method by which to make scaffolds for vascular tissue engineering purposes. Electrospinning is an attractive fabrication technique due to the tailorability of electrospun structures through changing any of the several parameters inherent to the process. An in-house electrospinning setup has been used in the BVM lab since 2009 to fabricate scaffolds of PLGA for Blood Vessel Mimic experiments, with the larger goal of BVMs being to characterize the cellular response of vascular tissues to various devices and environments. Additionally, many prior research efforts have focused on characterizing scaffold mechanical properties, investigating scaffold degradation, and finding the optimal parameters to reduce scaffold fiber diameter.

There were several aims to this thesis, all of which contributed towards the goal of improving the consistency, performance, and versatility of the BVM electrospinning technique. They were:

- 1) Improve scaffold characterization by comparing two techniques for fiber diameter measurement and implementing a technique for pore area measurement.

- 2) Reduce scaffold fiber diameter and pore area by investigating humidity and solvent composition as electrospinning parameters.
- 3) Reduce process variability by developing a less ambiguous electrospinning protocol.
- 4) Improve scaffold consistency and use by understanding and reducing PLGA scaffold shrinkage.
- 5) Identify and evaluate more flexible polymers as potential alternatives for electrospun BVM scaffolds.

For Aim 1, the methods by which fiber diameter had been characterized in the BVM lab was considered slow and potentially subject to operator error, and so this method was compared to an automated measurement method using the same ImageJ software on several SEM images of PLGA scaffolds, reference material, and images from literature. A protocol was also developed to measure pore area, as one had not been widely researched or accepted in the BVM lab. This was also compared to an automated method using the same gamut of images and measurement method characterization. The outcome of this work showed that the manual fiber diameter measurement method was more accurate compared to a known value and more accurately replicated results published past BVM lab theses, and is recommended for future BVM lab use. The pore area measurement tests were less conclusive, however they suggested that both manual

and DiameterJ methods could be used as a relative, comparative measure of pore area within the BVM lab. The manual pore area measurement method was ultimately recommended due to the ease with which parameters can be altered in the future to accommodate new findings and more accurate methods.

Many previous attempts to improve PLGA scaffold fiber diameter have been focused on altering the parameters directly related to the electrospinning setup, such as gap distance, flow rate, and applied voltage. However, literature sources suggested that significant reductions in fiber diameter can be achieved by changing either environmental conditions or solvent compositions, and so for Aim 2 several PLGA scaffolds were spun with either varying relative humidity values or varying concentrations of acetone and chloroform mixtures, and were characterized using the newly vetted fiber diameter and pore area measurement techniques. While no difference in fiber diameter was found between high and low relative humidity samples, the results of the solvent test were conclusive and promising. Increasing concentrations of acetone led to noticeable decreases in fiber diameter and pore size and triggered the onset of beaded fiber formation. The 1:1 chloroform:acetone ratio at 17wt.% PLGA concentration was found to produce the smallest fibers without the presence of beads.

With extensive use of the BVM lab electrospinner it became obvious that certain aspects of the current protocols could be improved to reduce chances of operator deviation from the intended procedure and to reduce variability inherent in the current

electrospinning setup. For Aim 3, alterations to the solution measurement methods, placement of components within the electrospinning setup, and removal and use of scaffolds were compared with the previously-used setup and deemed to have reduced electrospinning variability and improved usability. Following these comparisons, new solution mixing and electrospinning protocols were generated (Appendix G,H).

For Aim 4, the issue of scaffold shrinkage was pursued in-depth, the cause was identified, and a stress-relieving solution was proposed to reduce shrinkage during BVM setups. Specifically, a heat treatment step of 3 hours at 55 °C and a 70% ethanol solution soak for 1 hour were found to noticeably reduce scaffold shrinkage compared to a control sample. While both appeared suitable for future use in the BVM lab, the heat treatment method was ultimately recommended for future use due to its ability to treat several scaffolds at once without consuming lab supplies and because it is ready to use directly after treatment.

Finally, for Aim 5, 2 flexible polymer types were selected and electrospun in a preliminary study with the intention of achieving comparable fiber diameter results to current PLGA scaffolds and to produce an electrospun scaffold that could elastically conform to more tortuous BVM bioreactor designs. Both polymers were successfully electrospun with several iterations of the standard BVM electrospinning parameter set, however only PCL was able to form a consistent, fibrous scaffold that could be successfully removed from its mandrel. PCL was compared to both electrospun PLGA

and ePTFE tubing in a kink radius test described by ISO 7198. PCL was unable to bend without kinking, however its success as an electrospinnable polymer with morphology similar to that of PLGA was promising. This led to the recommendation that PCL research continue and that it be considered for use in place of PLGA in straight tube BVM setups due to its low cost and comparable electrospinning performance.

7.2 Challenges and Limitations

As with any research endeavor, there were multiple challenges faced while conducting the experiments described in this thesis. Several of the larger challenges will be described and discussed below.

7.2.1 Pore Area Measurements

While a pore area method was described, tested, and compared to another method as well as literature results, it is still unclear as to what constitutes a viable pore from the perspective of a cell. As such, the measurement of pore area in the BVM lab will primarily serve as a comparative tool with which electrospinning operators can evaluate the results of changes within the protocol and with which BVM results can be correlated.

7.2.2 Environmental Conditions

Qualitative experiences between BVM electrospinning operators and published literature both point to ambient environmental conditions as having a noticeable impact

on scaffold morphology, however the attempts to regulate temperature and humidity in this thesis were largely unsuccessful. This is believed to have been caused by the relatively large electrospinning chamber that it not sealed from the laboratory environment and is located within an operating fume hood. The ambient environment outside the chamber and constant flow of air around and possibly through the chamber could significantly hamper any attempts to regulate environmental conditions in the current state of the electrospinning setup. Suggestions for future work will be detailed in the section 7.3.1.

7.2.3 Chloroform Expiration and Electrospinner Refurbishing

Partway through the execution of this thesis, it was discovered that the chloroform used for electrospinning had expired more than 1 year ago. It was also discovered at this time that the method for chloroform extraction from the bottle was being performed improperly, and thus led to significant exposure of the stock solution to the ambient environment. This was assumed to have resulted in the absorption of water into the chloroform, altering its physical and electrical properties. Switching to new chloroform and chloroform extraction methods still did not produce results comparable to those generated in the thesis of Toni Pipes, results that have become the pinnacle of scaffold morphological characteristics in the BVM lab. This began a long process to investigate the remaining sources of inconsistency between current techniques and those performed in the 2014, which ultimately led to the discovery that the electrical connection between the electrospinning collector and the power supply was nonexistent, and essentially meant that the mandrel was not being grounded through a controlled source. Furthermore, other

uncovered issues with the spinner resulted in several modifications by BVM lab members and electrospinning collector manufacturers; Each scaffold spun on the system while in the process of being repaired could not be trusted to yield reliable results. In the pursuit of reducing electrospinning variability, the electrospinning situation in the BVM lab was made quite unpredictable for several months.

7.2.3.1 Old Electrospinning Collector

Investigating the steady increase in fiber diameter results produced in the BVM lab since the thesis of Toni Pipes was published led to the discovery that there was no consistent electrical connection between the mandrel and power supply during electrospinning, meaning that the buildup of charges caused by the depositing polymer jet could not be dispelled properly, reducing the attraction between mandrel and subsequent polymer jets during a given spin. The following is a condensed summary of events and attempted solutions regarding the pursuit of a properly functioning electrospinning collector.

- The internal collector wire connecting the mandrel to the port leading to the power supply displayed an “Overload” resistance reading, indicating a lack of electrical connection.
- Several new wires were researched and purchased, only to find that they did not properly interface with the collector.
- The manufacturer sent a refurbishing kit that included all the parts required to renovate the electrospinning collector to their current grounding wire design; the

frame of the spinner could not accommodate the requirements of the kit. At this time, another electrospinner collector was ordered and received to serve as a backup.

- The spinner was sent back to the manufacturer for refurbishment. The new spinner was used during this time.
- The old spinner was received and appeared to be working properly, and displayed a resistance value between the mandrel and power supply of approximately 8 k Ω , near the suggested value of the manufacturer.
- After some time the same resistance measurement was made, however the resulting value is above 100 k Ω . The solution to this issue was being pursued at the time of writing.

7.2.3.2 New Electrospinning Collector

A new electrospinning collector was ordered during the time in which the old collector was experiencing electrical connectivity issues to serve as the primary electrospinner in the meantime and to act as a backup for longterm use in the BVM lab. However, it experienced issues of its own during this time:

- Upon receipt of the new spinner it was noted that the alignment of the frame was not centered, causing uneven polymer collection along the length of the mandrel. This was due to an improperly machined component, and a new one was shipped from the manufacturer.

- Additionally, in comparison with the refurbished spinner prior to other resistance measurement troubles, it was found that the electrical connection between a mandrel and the grounding port out to the power supply would fluctuate across large ranges of resistance values (~10 to
- The new collector was shipped to the manufacturer who discovered the source of an incomplete connection between the grounding wire and the motor that spins the mandrel housing. This, along with the alignment issue, was fixed and shipped back to Cal Poly.
- Once received, the new collector displayed resistance values within range of what is suggested by the manufacturer, ~8 k Ω . At the time of writing the new spinner was in working order by all accounts.

7.3 Future Work

Because this thesis focused on many different areas of the electrospinning process, it also resulted in many issues that could quite easily become the focuses of future work.

7.3.1 Reducing Fiber Diameter

The results published in this thesis regarding changes in fiber diameter with solvent composition alterations are very promising for the goals of the BVM lab, and may be expanded upon quite readily. While it was shown that the inclusion of acetone into the standard PLGA-chloroform solution can result in a drastic decrease in fiber

diameter, these scaffolds have not been tested in a BVM setting; This may be the next logical step in confirming that the morphologies observed here are beneficial for cell adhesion and proliferation. Regarding the scaffold fabrication side: acetone and chloroform were simply two solvents that had been combined in literature previously and were readily available to the BVM lab, however they are far from the only solvent combinations published in electrospinning literature before. Other solvent combinations in various ratios could be tested with PLGA and the current electrospinning protocol with ease. Additionally, several sources have cited instances of further improvement of results by decreasing the formation of beads through incorporation of surfactants or ionic compounds into the electrospinning solution. Because smaller fibers tend to form with low viscosity and because beads commonly form due to a mismatch of high surface tension with low viscosity, reducing surface tension and bead formation may allow one to further decrease viscosity and thus fiber diameter^{110,128,135,189}.

7.3.2 Process Variability

While many sources of variability in the BVM electrospinning process were considered in this thesis, and Appendix G and H include improved and more reproducible protocols, several additional sources of variability exist. Perhaps the most important is the inability to regulate environmental parameters such as temperature and humidity, which means there is still significant uncertainty as to whether these factors affect the electrospinning procedure. Constructing a smaller isolated, hermetically sealed chamber in which electrospinning could be performed at various temperature and humidity values

may be advantageous to investigate the effect of these parameters and to decide whether they could be implemented into the main electrospinning system.

7.3.3 Scaffold Shrinkage

The methods and experiments described in this thesis suggest that both heat treatment and ethanol treatment prevent some amount of shrinkage, however more work could be done to optimize the stress-relief process. Scaffolds still experienced some shrinkage after treatment, which may be resolved by stress-relieving for longer periods of time or at more extreme conditions such as higher temperatures or higher ethanol concentrations. Additionally, it may be the case that the time periods described are too long to be practical, and may be unnecessary. Therefore, work could also be done to investigate the ideal conditions for integration with the BVM setup protocol while still providing adequate shrinkage mitigation. It is advised that the BVM lab pursue the relationship between the degree of shrinkage mitigation and the time and intensity of stress-relieving treatments to fully understand the capabilities and limitations of the process.

7.3.4 Alternative Electrospinning Materials

While both PCL and the two tPU materials selected as possible flexible alternatives to PLGA ultimately did not achieve the goal of successfully forming scaffolds that could be used in tortuous BVM bioreactor designs, the fact that the BVM electrospinning system can readily accommodate other materials is promising. The

materials selection effort presented herein relied on CES to provide a list of acceptably flexible materials, however it is possible that published research regarding vascular tissue engineering from other institutions may provide ideas for other flexible polymers to spin. CES also did not consider the concept of blending polymers in solution, a common technique in electrospinning research. It is still the case that the current PLGA scaffolds are too stiff to accurately replicate the mechanical properties of native vessels, and so if work on more tortuous bioreactor designs is to continue then investigating a flexible polymer alternative will be necessary. Table XLIII contains a concise summary of all recommended future work to build upon the results of this thesis:

Table 43. Summary of All Suggested Future Work

Future Work	Approach
Improve pore area measurement technique	Continue to refine the pore area measurement protocol, identifying sources of variability and parameters that will accurately and consistently provide meaningful pore area data.
Further reduce PLGA fiber diameter	Use other solvents and solvent combinations as described in literature to create scaffolds for BVM use
Investigate the effect of reduced fiber and pore size on BVM results	Use pure acetone and chloroform:acetone scaffolds (or others with comparable) of a 1:1 ratio in BVM setups and observe trend in cell coverage and response.
Improve Electrospinning environmental control	Design and construct a smaller, sealed electrospinning chamber in which temperature and humidity can be controlled and investigate the effects of both.
Compare PCL and PLGA in a BVM setup	Develop parameter sets that produce PLGA and PCL scaffolds of equivalent fiber diameter and/or pore area and test both in a BVM setup to observe any differences.
Pursue PCL as an electrospinning polymer	If PCL results from the aforementioned BVM test are promising, attempt similar fiber reduction techniques through manipulation of solvent composition on PCL.
Find a suitable flexible electrospinning polymer	Review literature on flexible tissue engineering scaffolds and attempt to electrospin the flexible materials described, characterizing them using the same ISO 7198 standard.

7.4 Conclusion

In conclusion, the characterization, overall performance, variability, and possibility for increased versatility of the BVM electrospinning setup was investigated. The current fiber diameter measuring method was shown to be more accurate and consistent compared to DiameterJ; A preliminary pore area measurement method was also developed and compared to DiameterJ, however neither were conclusively deemed superior from one another. Fiber diameter was shown to decrease significantly in PLGA scaffolds with the use of alternative solvent compositions. The solution mixing and electrospinning protocols were improved to reduce variability and improve user experience. Attempts to regulate environmental conditions were not successful, however methods described to reduce shrinkage caused by ethanol and elevated temperature exposure of electrospun PLGA scaffolds were found to have a positive effect. Finally, 2 flexible polymers were shown to produce an electrospun scaffold, however thermoplastic polyurethane could not be successfully removed from the mandrel and PCL experienced noticeably kinking when bent any appreciable amount. Ultimately these efforts contributed towards decreasing average PLGA scaffold fiber diameter, reducing sources of variability in the BVM electrospinning process, and provided several starting points for further research into these areas.

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APPENDICES

APPENDIX A: IMAGEJ PROTOCOL FOR FIBER DIAMETER MEASUREMENT

1. Find or create a 4x4 circle grid with dimensions equal to that of the working area of the SEM images to be measured.

4x4 circle template of 1280x960 pixels

SEM image at 1280x1040 pixels
(effective area of 1280x960).

2. Open ImageJ software; the following should appear:

3. Open the SEM image(s) of interest as well as the circle template through ImageJ (File>Open or Ctrl+O).
4. Use the Enhance Contrast ImageJ tool to increase pixel saturation in the SEM image.
 - a. Click to make the desired image the active window then select Process>Enhance Contrast and input “15%” in the Saturated Pixels box, leaving the remaining selections unchecked.

b. The image should have noticeably more contrast:

Before and after 15% pixel saturation using ImageJ Enhance Contrast tool.

5. Overlay the circle template image onto the scaffold SEM image using the ImageJ Overlay tool (Image>Overlay>Add Image) and select the circle template image.
 - a. Ensure that the location settings are both set to “0”, opacity is set to 100%, and that the “Zero transparent” box is checked.
 - i. This will ensure the template is properly aligned and visible while removing all the black from the image.

- b. The resulting image should appear as such:

6. Select the Line tool on the ImageJ toolbar and trace the length of the scale bar of the SEM image.

Select "Set Scale" (Analyze>Set Scale) and input the length of the scale bar in to automatically convert pixel measurements to microns.

7. Use the Line tool to measure one fiber in all circles, pressing the "t" key to save the measurement in the ImageJ ROI Manager before measuring the next fiber.

- a. Select fibers nearest to the center of each circle of which the entire diameter of the fiber can be measured. Fibers closest to the luminal surface are often the easiest to measure, assuming they fit the previous criteria.

8. After all measurements are recorded, highlight all ROI Manager entries and select the “Measure” option to generate a data table of line dimensions.

	Perim.	Angle	Length
1	29.8329	-13.5704	29.8329
2	15.5563	45.0000	15.5563
3	26.2488	-40.3645	26.2488

- a. Other measurement options can be obtained by selecting Results>Set Measurements.
9. Save the Results data table along with a copy of the measured image and repeat steps 3 through 10 for all SEM images of interest.

APPENDIX B: DIAMETERJ PROTOCOL FOR FIBER DIAMETER AND PORE AREA MEASUREMENTS

1. Install the DiameterJ plugins for ImageJ:
https://imagej.net/DiameterJ#Download_Link
2. Open ImageJ along with an SEM image to be measured
3. Select the Line tool on the ImageJ toolbar and trace the length of the scale bar of the SEM image.

Select "Set Scale" (Analyze>Set Scale) and input the length of the scale bar in to automatically convert pixel measurements to microns.

4. Select the segmentation plugin for DiameterJ: Plugins>DiameterJ>DiameterJ Segment
 - a. To crop the SEM information bar from the image, insert the correct sizes into the cropping field boxes.
 - b. Select Traditional, Stat. Region Merged, and Mixed segmentation algorithms.
 - c. Select "Yes" when asked "Do you want to analyze more than one image?".

- d. Select the appropriate location for DiameterJ to search for your images; it will segment all images within this folder.
- e. The segmentation plugin will create 3 folders within the selected folder: Best Segmentation, Montage Images, and Segmented Images

5. Select the Montage Images folder and compare all the segmentation options to the original image, located in the top-left corner of the montage image.

- a. Use the full sized images in the Segmented Images folder to directly compare a few images for difference in smaller features if necessary.
 - b. Choose the “best” segmented image based on its similarity to the original in terms of features displayed as well as their size/thickness, and avoid images with areas that are completely filled in with white and images with lots of fiber “loose ends” that disappear instead of connect with the rest of the fiber network.
6. Once the most accurate segmented image is identified, move it into the Best Segmentation folder and return to ImageJ.
7. In ImageJ, select the DiameterJ image analysis plugin:
Plugins>DiameterJ>DiameterJ 1-018

DiameterJ Options

Orientation Analysis

Orientation Analysis:

*Note: Directionality is much slower

Automated Unit Conversion

Do you want DiameterJ to convert all output from pixels to real units?

☒ Yes ☐ No

Length of scale bar Pixels

Length of scale bar Microns

Identify Specific Radius Locations

Do you want to identify the location of a specific radius?

☐ Yes ☒ No

Min. Fiber Radius Pixels

Max. Fiber Radius Pixels

Batch Processing

Do you want to analyze more than one image?

☐ Yes ☒ No

Do you want DiameterJ to combine analysis from all images?

☐ Yes ☒ No

- Select “None” for Orientation Analysis
- Include the pixel-to-micron ratio provided by measuring the image scale bar in Step 3 and select “Yes” to convert all units to microns.
- Select a specific radius range to be identified, if desired.
- Select the final 2 options based on preference and whether multiple segmented images were selected*.

*An error sometimes occurs in which the analysis will not start if the “yes” option is not selected for the prompt “Do you want to analyze more than one image?”. Select “yes” if this issue is encountered.

- Select “Ok” and then select the Best Segmentation folder when prompted; the analysis will begin.

8. The Best Segmentation folder now contains folders for Combined Files, Diameter Analysis Images, Histograms, and Summaries along with the original segmented image.

- a. The raw data values for fiber radius and pore area are located in the Histograms folder, and are labeled “SegmentedImageName_Radius Histo” and “SegmentedImageName_Pore Data”, respectively.

- b. These values are summarized in the Excel document located in the Summaries folder.
9. Repeat Steps 2-8 for all SEM images to be measured.

APPENDIX C: IMAGEJ PROTOCOL FOR PORE AREA MEASUREMENT

1. Open ImageJ software; the following should appear:

2. Open the SEM image(s) of interest through ImageJ (File>Open or Ctrl+O)
3. Use the Enhance Contrast ImageJ tool to increase pixel saturation in the SEM image.
 - a. Click to make the desired image the active window then select Process>Enhance Contrast and input “15%” in the Saturated Pixels box, leaving the remaining selections unchecked.

- b. The image should have noticeably more contrast:

Before and after 15% pixel saturation using ImageJ Enhance Contrast tool.

4. Select the Line tool on the ImageJ toolbar and trace the length of the scale bar of the SEM image.

Select “Set Scale” (Analyze>Set Scale) and input the length of the scale bar in to automatically convert pixel measurements to microns.

5. Press Shift+f to use the “Flatten” tool in ImageJ. This preserves the contrast enhancement.
 - a. After flattening an image it must be converted back into an 8-bit version for pore area measurements: select Image>Type>8-bit from the ImageJ tool bar.

6. Select the entire image using Ctrl+a and move the area selection box to exclude the information at the bottom of the SEM image and press Ctrl+x to crop the image.

7. Select Image>Adjust>Threshold (or Ctrl+Shift+T) to open the manual thresholding control panel.

Thresholding control panel

SEM image during thresholding

- a. Select a pixel color limit that includes all luminal fibers and properly fills all apparent pores with red.
 - b. Select the "Set" option and then select "Ok" on the following screen.
8. After thresholding, the now-highlighted pores can be measured using the ImageJ "Analyze Particles" tool. Select Analyze>Analyze Particles, and select the desired pore area range, units, circularity parameters, outline or mask generated (if any), and various other settings regarding the method of pore area measurement.

9. The Analyze Particles tool produces a Results table; confirm that it has produced area values in the desired units and save the table.
 - a. If an outline or mask was generated it can be saved through the ImageJ toolbar or by pressing Ctrl+s.
10. Repeat steps 2 through 9 for all SEM images to be measured.

APPENDIX D: ORIGINAL BVM PLGA SOLUTION MIXING PROTOCOL

Purpose: The purpose of this SOP is to make a solution of PLGA in chloroform to be used for scaffold electrospinning (SOP5311).		
Approx. Time: 30 min Abbreviations: 1. PLGA - poly(lactic-co-glycolic) acid Reminders: 1. Always keep solution wrapped in foil and limit exposure to light. 2. Chloroform evaporates quickly—be sure to work quickly and cap things off. 3. Record the following in lab notebook: mass of PLGA used and time vial was placed on shaker 4. This protocol makes a 15wt% PLGA-Chloroform solution Materials: 1. Aluminum Foil 2. 20 mL vial 3. Chloroform 4. PLGA crystals 5. 10 mL Syringe 6. Scale 7. Shake table	Procedure: <u>A. Solution Preparation</u> 1. Assure lab hygiene protocol has been followed 2. Take container of PLGA out of freezer and allow to thaw for approx 10 min ❶ 3. Wrap 20 ml vial in aluminum foil ❷ 4. Weigh the correct amount of PLGA with a scale ❸ 4.1. Be sure to zero the scale with the tray first 4.2. Obtain 0.7835 grams +/- .0001 5. Pour the weighed PLGA crystals in the 20 ml vial 6. Take chloroform and vial into the chemical hood 7. Measure 3 ml of chloroform using a syringe and put into vial of PLGA 7.1. Immediately cap the vial 8. Place vial on the shaker table 9. Secure the vial on the shaker table using tape ❹ 10. Write date, initials, and time started on the tape and in your lab notebook 11. Turn on shake table at a setting of 4 for approx 24 hours. 12. Congratulations! You have made a PLGA-Chloroform solution.	<div style="display: flex; justify-content: space-around;"> <div style="text-align: center;">❶ </div> <div style="text-align: center;">❷ </div> </div> <div style="text-align: center;">❸ </div> <div style="text-align: center;">❹ </div>
Revision History:		
A	Toni Pipes	Fall 2013
B	Jakub Truty	Spring 2014

APPENDIX E: ORIGINAL BVM ELECTROSPINNING PROTOCOL

Purpose: The purpose of this SOP is to guide the user in electrospinning a tubular scaffold from PLGA.		
Approx. Time: 1 hour	Procedure: <u>A. Preparation</u>	1
Abbreviations: 1. PLGA – Poly Lactic Glycolic Acid	<ol style="list-style-type: none"> 1. Assure lab hygiene protocol has been followed 2. Continuing from SOP5310, take PLGA-Chloroform solution off shake table <ol style="list-style-type: none"> 2.1. Record the time the solution is taken off 3. Completely wipe down hood and electrospinner with IPA 4. To clean each mandrel: <ol style="list-style-type: none"> 4.1. Sand mandrel with 1200 grit sandpaper by wrapping the sandpaper around the mandrel and using a twisting motion 4.2. Wipe down with a paper towel and IPA until no residue is visible on the paper towel 5. Load mandrel onto the electrospinner by first inserting the distal end, followed by the proximal end. 1 <ol style="list-style-type: none"> 5.1 The pins on the proximal end fit into slots on the electrospinner. 5.2 After fitting both ends, adjust length by turning knob on distal end of electrospinner until a snug fit is achieved. 	3
Reminders: <ol style="list-style-type: none"> 1. WARNING: This process utilizes high voltages. 2. To use the electrospinner, you must be trained and approved by a faculty member and always use necessary protections 3. After cleaning the mandrels, do not touch them anywhere except the pins 4. Dispose any material that came in contact with chloroform into the hazardous waste bucket 5. The negative electrode is red and the grounding electrode is black 	<ol style="list-style-type: none"> 6. Using a syringe, take up 3 ml of PLGA-chloroform solution from the 20 ml vial. <ol style="list-style-type: none"> 6.1. Tap on the syringe to let any bubbles loose 2 6.2. Depress plunger and leech solution into a paper towel to get rid of any air 3 7. Attach needle tip to syringe 8. Load syringe into the unit by inserting the needle through the hole in the plastic housing and secure tightly with the black clamp 4 9. Attached negative electrode to needle of syringe 10. Place electrospinner directly over the 10 inch mark 11. Plug in unit and turn on by flipping the switch on surge protector so the button is green 5 12. Ground the mandrel by rubbing ground electrode along the length of the mandrel and along every side 6 <ol style="list-style-type: none"> 12.1. Insert ground electrode back into the electrospinner 	4

<p>Materials:</p> <ol style="list-style-type: none"> 1. PLGA-Chloroform solution in 20 ml vial 2. Mandrel 3. 1200 grit sandpaper 4. 10 ml syringe 5. 18 gauge beveled blunt needle 6. Electrospinner 	<ol style="list-style-type: none"> 13. On the syringe unit, place end block so it gently touches the end of the syringe 14. Press select twice to input data in the following steps 15. Input volume (3.5 ml) and, press select, and input flow rate (5.5 ml/hr) into the syringe unit 16. Press select again to show volume being ejected 17. Adjust slide and rotate settings on unit below the syringe pump to 3 and 6, respectively. 18. Press Run/Stop Button on syringe pump to start <p style="text-align: center;"><u>B. Electrospinning Operation –</u></p> <ol style="list-style-type: none"> 1. Wait for a bead to form on the tip of the syringe needle <ol style="list-style-type: none"> 1.1. Take note of the temperature, humidity, and bead formation with the volume ejected displayed on the syringe pump unit 2. When the bead forms, turn on the voltage and slide/rotate switches. 7 <ol style="list-style-type: none"> 2.1 Adjust voltage to -12 kV. 2.2 Take notes on the Taylor Cone or any unusual observations 3. Run the electrospinner until syringe is empty <ol style="list-style-type: none"> 3.1. The mandrel should turn white as polymer attaches 3.2. If the pump unit reaches inputted volume but syringe is not empty, increase the volume on the pump before it reaches 3.5 ml by pressing select twice and using arrows to increase or decrease volume. Press select twice to go back to current status screen. 4. Turn off the voltage supply by flipping the switch, the slide/rotate by flipping two switches, turn off the surge protector (green button), and unplug from electric outlet 5. Move end block away from syringe plunger and take off the negative electrode from the needle 6. Remove syringe needle and throw away in sharps container 7. Throw away syringe in the hazardous waste bucket 8. Unload mandrel from electrospinner and place in desiccator <ol style="list-style-type: none"> 8.1. Note the time the scaffold + mandrel were placed in the desiccator 	<p style="text-align: center;">5</p> <p style="text-align: center;">6</p> <p style="text-align: center;">7</p> <p style="text-align: center;">8</p>
<p>Revision History:</p>		
A	Toni Pipes	Fall 2013
B	Jakub Truty	Spring 2014

APPENDIX F: STANDARD OPERATING PROCEDURE FOR MIXING ACETONE
AND CHLOROFORM FOR THE PURPOSES OF PLGA ELECTROSPINNING

Standard Operating Procedure for Laboratory Processes

Chemical Name or Process:

Electrospinning PLGA solution onto a bare metal (303 stainless steel) cylindrical mandrel.

Purpose:

To investigate the effects of solvent composition on electrospun scaffold fiber diameter and average pore size using mixtures of acetone and chloroform to dissolve PLGA.

Potential Hazards/Toxicity:

Acetone:

Caution: Flammable and toxic.

Chloroform:

Caution: Toxic, possible carcinogen.

Acetone+Chloroform Mixture:

In addition to the standard hazards of Acetone and Chloroform, the combination of the two in the presence of a basic environment will undergo a highly exothermic condensation reaction to form 1,1,1-trichloro-3-hydroxy-3-methylketone. This reaction is known to be violent enough to shatter glass containers (from Bretherick's, attached).

Isopropyl Alcohol (IPA):

Caution: Flammable and toxic.

Engineering Controls:

All work to be performed in a fume hood

Personal Protective Equipment (PPE)-

Hand Protection:

Nitrile gloves will be used with isopropyl alcohol, acetone, chloroform, and any mixtures of the acetone and chloroform.

Eye Protection:

Splash protection goggles will be used when handling acetone, chloroform, and any mixtures of the two substances, and with isopropyl alcohol.

Skin and Body Protection:

Lab personnel working with the chemicals need to wear full-length pants or its equivalent, closed-toe footwear with no skin being exposed, and a lab coat.

Hygiene Measures:

Wash hands after working with the hazardous substances and when leaving the lab/shop. Gloves must be inspected prior to use. Use proper glove removal technique (without touching glove's outer surface) to avoid skin contact with this product. Dispose of contaminated gloves after use

Respirators may be required under any of the following circumstances:

- As a last line of defense (i.e., after engineering and administrative controls have been exhausted).
- When Permissible Exposure Limit (PEL) will or may be exceeded, or the airborne concentration is unknown.
- Regulations require the use of a respirator.
- There is potential for harmful exposure due to an atmospheric contaminant (in the absence of PEL)
- As PPE in the event of a chemical spill clean-up process

Prior to obtaining a respirator, an exposure assessment of the process or procedure must be conducted. If respiratory protection is required, then lab personnel must obtain respiratory protection training, a medical evaluation, and a respirator fit test through EH&S. This is a regulatory requirement.

First Aid Procedures for Chemical Exposures

If inhaled:

Evacuate the victim to a safe area as soon as possible. Loosen tight clothing such as a collar, tie, belt or waistband. If breathing is difficult, seek medical attention. If the victim is not breathing, perform mouth-to-mouth resuscitation. **WARNING:** It may be hazardous to the person providing aid to give mouth-to-mouth resuscitation when the inhaled material is toxic, infectious or corrosive. Seek immediate medical attention.

In case of skin contact:

In case of contact, immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Cold water may be used. Wash clothing before reuse. Thoroughly clean shoes before reuse. Get medical attention, as necessary.

In case of eye contact:

Immediately flush eyes with plenty of water for at least 15 minutes. Check for and remove any contact lenses. Get medical attention.

If swallowed: Do NOT induce vomiting. Never give anything by mouth to an unconscious person. Rinse mouth with water. Consult a physician or a poison center if you feel unwell.

Special Handling and Storage Requirements

Acetone and Isopropyl Alcohol:

Avoid contact with skin and eyes. Avoid inhalation of vapour or mist. Use explosion-proof equipment. Keep away from sources of ignition - No smoking. Take measures to prevent the buildup of electrostatic charge. Keep container tightly closed in a dry and well-ventilated place. Containers which are opened must be carefully resealed and kept upright to prevent leakage.

Chloroform:

Wear personal protective equipment. Use only under a chemical fume hood. Do not breathe vapors or spray mist. Do not get in eyes, on skin, or on clothing. Do not ingest. Keep away from open flames, hot surfaces and sources of ignition. Keep containers tightly closed in a dry, cool and well-ventilated place. Keep away from direct sunlight. Store under an inert atmosphere. Protect from moisture.

Spill and Accident Procedure

Chemical Spill Dial 911 and 756-6661

Spill – Assess the extent of danger. Help contaminated or injured persons. Evacuate the spill area. Avoid breathing vapors. If safe, confine the spill to a small area using a spill kit or absorbent material. Keep others from entering contaminated area (e.g., use caution tape, barriers, etc.).

Small (<1 L) – If you have training, you may assist in the clean-up effort. Use appropriate personal protective equipment and clean-up material. Double bag spill waste in plastic bags, label and arrange hazardous waste pick-up.

Large (>1 L) – Evacuate spill area. Dial 911 and EH&S at 756-6661 for assistance. Remain available in a safe, nearby location for emergency personnel.

Chemical Spill on Body or Clothes – Remove clothing and rinse body thoroughly in emergency shower for at least 15 minutes. Seek medical attention. *Notify supervisor, advisor or P.I. immediately.*

Chemical Splash Into Eyes – Immediately rinse eyeball and inner surface of eyelid with water from the emergency eyewash station for a minimum of 15 minutes by forcibly holding the eye open. Seek medical attention. *Notify supervisor, advisor or P.I. immediately.*

Medical Emergency Dial 911 or 756-6661

Life Threatening Emergency, After Hours, Weekends And Holidays – Dial 911

Note: All serious injuries must be reported to Supervisor/PI within 8 hours. Note: Any and all loss of consciousness requires a 911 call

Non-Life Threatening Emergency –

- Students: Seek medical attention at the campus Health Center **M, T, Thu, Fr 8:00 am – 4:30 pm and W 9:00 am – 4:30 pm**
- Emergency Medical services in the community are available at any time at hospital emergency rooms and some emergency care facilities.

All injuries must be reported to PI/Supervisor immediately and follow campus injury reporting. Follow procedures for reporting of student, visitor injury on the EH&S website at: <http://afd.calpoly.edu/riskmgmt/incidentreporting.asp>

- Paid staff, students, faculty: seek initial medical attention for all non-life threatening injuries at:
 - MED STOP, 283 Madonna Road, Suite B (next to See's Candy in Madonna Plaza)
(805) 549-8880 Hours: M-F 8a - 8p; Sat/Sun 8a - 4p
 - **After MED Stop Hours:** Sierra Vista Hospital Emergency Room
1010 Murray Avenue (805) 546-7651, Open 24 hours

All injuries must be reported to PI/Supervisor immediately and follow campus injury reporting for employee injuries (Workmen's Comp.). Follow procedures on the EH&S website at: <http://afd.calpoly.edu/riskmgmt/incidentreporting.asp>

Needle stick/puncture exposure (as applicable to chemical handling procedure) – Wash the affected area with antiseptic soap and warm water for 15 minutes. For mucous membrane exposure, flush the affected area for 15 minutes using an eyewash station. Seek medical attention. *Note: All needle stick/puncture exposures must be reported to supervisor, advisor or P.I. and EH&S office immediately.*

Decontamination/Waste Disposal Procedure

Store all contaminated waste separate from standard chloroform and acetone waste containers.

General hazardous waste disposal guidelines:

Label Waste

- Affix a hazardous waste tag on all waste containers as soon as the first drop of waste is added to the container. Generic waste labels can be found here:
http://afd.calpoly.edu/ehs/docs/hazwaste_label_template.pdf

Store Waste

- Store hazardous waste in closed containers, in secondary containment and in a designated location
- Double-bag dry waste
- Waste must be under the control of the person generating & disposing of it

Dispose of Waste

- Dispose of regularly generated chemical waste as per guidelines on EH&S website at:
http://afd.calpoly.edu/ehs/docs/csb_no6.pdf
- Prepare for transport for pick-up. Use secondary containment.

Call EH&S at 756-6661 for questions.

Empty Containers-

- Dispose as hazardous waste if container once held extremely hazardous waste (irrespective of the container size) A list can be found at: http://afd.calpoly.edu/ehs/docs/extremely_hazardous_wastes.pdf
- All other containers are legally empty once a concerted effort is made to remove, pour out, scrape out, or otherwise completely empty the vessel. These may be disposed of as recycling or common trash as appropriate.

Safety Data Sheet (SDS) Location

Online SDS can be accessed at: <http://siri.org/msds/index.php>

or MSDSOnline at: <http://hq.msdsonline.com/csuedusl/Search/Default.aspx>

Copy of SDS for Chloroform, Acetone, and Isopropyl alcohol are attached.

Protocol/Procedure (Add lab specific Protocol/Procedure here)

Electrospinning: Note: Be cautious with needles

A. Preparation

1. Put on determined personal protective equipment, such as appropriate gloves, eye wear, etc.
2. Bring Acetone and Chloroform out of storage to fume hood
3. Using two separate syringes, draw out 0.5 – 1.5 mL of each solvent (to ensure a 1:1 ratio of Acetone:Chloroform)
4. Combine syringe contents in a single vial 20mL vial.
5. Insert weighed amount of PLGA into vial (while still in fume hood).
6. Close vial and place on shaker table for 24-48 hours at a setting of 4 on table.
7. Take the solution off the shake table and record time
8. Completely wipe down hood and electrospinner with 70% IPA
9. Sand Mandrel with 1200 grit sandpaper by wrapping the sandpaper around the mandrel using a twisting motion
10. Wipe the mandrel with 70% IPA until all residue is gone
11. Load mandrel onto Electrospinner by first inserting the distal end, followed by the proximal end
12. The pin on the proximal end fit into the slots on the electrospinner
13. After fitting both ends, adjust length by turning knob on the distal end of the electrospinner until a snug fit is achieved
14. Using syringe, take up full amount of Acetone-Chloroform-PLGA solution from the 20mL vial
15. Tap on syringe to let any bubbles loose
16. Depress plunger to leech solution into a paper towel/inert material to let out any air
17. Attach needle tip to syringe

18. Load syringe into the unit by inserting the needle through the hole in the plastic housing and secure tightly with the black clamp
19. Attach negative electrode to needle of syringe
20. Place electrospinner over the 10 inch mark
21. Plug in unit and turn on by flipping the switch on surge protector so the button is green
22. Ground the mandrel by rubbing ground electrode along length of the mandrel and along every side
23. Insert ground electrode back into electrospinner
24. On the syringe unit, place end block so it gently touches the end of the syringe
25. Input volume (total mL draw into syringe plus .5 mL) and press select, then input flow rate (will vary between experiments) into the syringe unit
26. Adjust the slide and rotate settings (will vary between experiments)
27. Press run/stop on syringe pump to start

B. Electrospinning

1. Wait for bead to form on the tip of the syringe needle
 - a. Take note of the temperature humidity and bead formation with the volume ejected displayed on the syringe pump unit
2. When bead forms turn on the voltage and slide/rotate switches
 - a. Adjust voltage (will vary between experiments, likely between -10 and -20 kV)
 - b. Take notes on Taylor Cone or any unusual observations
3. Run electrospinner until syringe is empty
 - a. Increase volume output on syringe pump if the syringe is not empty at volume limit
4. Turn off voltage by flipping the switch, the slide/rotate by flipping two switches and turn off surge generator
5. Remove syringe needles and deposit in sharps container
6. Throw away syringe in appropriate hazardous waste container
7. Unload mandrel from electrospinner and place in desiccator for at least 48 hours to allow complete evaporation of residual solvent

NOTE:

Any deviation from this SOP requires approval from PI.

Date: 4/28/2017 **P.I. or Supervisor:** Kristen O'Halloran Cardinal

Documentation of Training (signature of all users is required)

- The Principal Investigator must ensure that his/her laboratory personnel have attended appropriate laboratory safety training or refresher training within the last one year.

- Training must be administered by PI or Lab Manager to all personnel in lab prior to start of work with particularly hazardous substance or newly synthetic chemical listed in the SOP.
- Refresher training will need to be provided when there is a change to the work procedure, an accident occurs, or repeat non-compliance.

I have read and understand the content, requirements, and responsibilities of this SOP:

Name	Signature	Date
Click here to enter text.		Click here to enter a date.
Click here to enter text.		Click here to enter a date.
Click here to enter text.		Click here to enter a date.
Click here to enter text.		Click here to enter a date.
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APPENDIX G: REVISED BVM PLGA SOLUTION MIXING PROTOCOL

Purpose: The purpose of this SOP is to make a solution of PLGA in chloroform to be used for scaffold electrospinning (SOP5311).

Approx. Time:
30 min

Abbreviations:
PLGA - poly(lactic-co-glycolic) acid

Reminders:

Always keep solution wrapped in foil and limit exposure to light.
Chloroform evaporates quickly—be sure to work quickly and cap things off.
Record the following in lab notebook: mass of PLGA used and time vial was placed on shaker
This protocol makes a 15wt% PLGA-Chloroform solution, however general equations are provided to alter concentration.

Materials:

Aluminum Foil
20 mL vial
Chloroform
PLGA pellets
10 mL syringe
Mass balance
Shaker table

Procedure:

A. Solution Preparation

Assure lab hygiene protocol has been followed.

Take container of PLGA out of freezer and allow to thaw for approx 10 minutes. ❶

In the meantime, wrap 20 ml vial in aluminum foil and label with tape. ❷

3.1. Include date (YYMMDD), initials, and contents.

Weigh the correct amount of PLGA with a mass balance. ❸

4.1. Be sure to zero the scale with the tray first.

4.2. In order to limit PLGA exposure to the environment, cap the bottle between weighing.

4.2. Obtain a PLGA mass of 0.7835 grams +/- 0.004.

Pour the weighed PLGA pellets in the 20 ml vial.

Obtain a chloroform bottle from the blue Corrosives cabinet and bring it, a syringe, a long needle with needle core, and the vial into the biological safety cabinet.

Remove the needle from its packaging and open the chloroform bottle to reveal a dark film. Pierce this film with the needle, remove the needle core, and attach the syringe. ❹

Key: M_t : total solution mass M_p : polymer mass V : solvent volume ρ : solvent density n : number of solvents M_s : combined solvent mass.

7.1. This is done to prevent chloroform degradation and water absorption. Do not remove the black film cap.

Measure 3 ml of chloroform using a syringe and eject into vial of PLGA.

8.1. Purge the air from the syringe by depressing the plunger while pointing the needle at a paper towel inside the hood.

8.2. Immediately cap the vial once chloroform has been ejected.

8.3. Dispose of the syringe and paper towel into the chlorinated waste stream container below the hood; sheath the needle and core and dispose in the sharps container, and cap the chloroform and return it to the Corrosives cabinet.

Place vial on the shaker table in 007-04.

Secure the vial on the shaker table using tape

5

Write date, initials, and time started on the tape and in your lab notebook

Turn on shake table at a setting of 4 for approx 24 hours.

Ensure that the dehumidifier is located inside the electrospinning enclosure and is turned on.

Congratulations! You have made a PLGA-Chloroform solution.

B. General Solution Concentration Equations

1. If attempting to create solutions of other concentrations or with different polymers and solvents, the following equations can be used:

$$M_t = M_p + (V_1 * \rho_1) + \dots (V_n * \rho_n)$$

$$M_s = V_1 * \rho_1 + \dots V_n * \rho_n$$

Revision History:

A	Toni Pipes	Fall 2013
B	Jakub Truty	Spring 2014
C	Evan Dowey	Summer 2017

APPENDIX H: REVISED BVM ELECTROSPINNING PROTOCOL

Purpose: To guide the user in electrospinning a tubular scaffold from a PLGA-Chloroform solution.		
Approx. Time: 1 hour	Procedure: <u>A. Preparation</u> <ol style="list-style-type: none"> 1. Assure lab hygiene protocol has been followed. 2. Continuing from SOP4320, remove electrospinning solution from the mixing table. <ol style="list-style-type: none"> 2.1. Record the time the solution is removed/total time mixed. 2.2. Also turn off and remove the dehumidifier from the electrospinning chamber. 3. Completely wipe down hood and electrospinner with IPA. 4. Clean each mandrel by wiping thoroughly with a paper towel wetted with IPA. <ol style="list-style-type: none"> 4.1. Use a twisting motion along length of mandrel. 4.2. Once cleaned, refrain from touching any part of the mandrel aside from the “t-shaped” pins. 5. Load mandrel into the electrospinner by first inserting the distal end, followed by the proximal end. 1 <ol style="list-style-type: none"> 5.1. The pins on the mandrel fit into slots on the proximal end of the electrospinner. 5.2. After fitting both ends, turn the threaded knob on the distal end of the electrospinner until a snug fit is achieved; be sure to engage the spring-loaded end. 	<div style="text-align: center;">1</div> <div style="display: flex; justify-content: space-around;"> <div style="text-align: center;">2</div> <div style="text-align: center;">3</div> </div>
Abbreviations: <ol style="list-style-type: none"> 1. PLGA – Poly(lactic-co-glycolic acid) 2. IPA – Isopropyl Alcohol 		
Reminders: <ol style="list-style-type: none"> 1. WARNING: This process utilizes high voltages. 2. To use the electrospinner you must be trained and approved by a faculty member and always use necessary protections. 3. Dispose any material that contacted chloroform solutions (<u>except sharps/needle tips</u>) into the hazardous waste container. 4. The negative wire is red and the grounding wire is white. 5. Limit exposure to light: chloroform will degrade. Do not use hood light. 		

<p>Materials:</p> <ol style="list-style-type: none"> 1. PLGA-Chloroform solution in 20 ml vial. 2. Mandrel 3. 10 ml syringe 4. 18 gauge beveled blunt needle 5. Electrospinner 6. IPA spray bottle and paper towels 	<ol style="list-style-type: none"> 6. Ground the collector and mandrel by plugging the white cable into the grounding port on the rear of the electrospinner. 7. Place the electrospinner over the 10 inch mark on the floor of the enclosure. 8. In front of the controller box and syringe pump, lay down paper towel and take up the electrospinning solution into a syringe. <ol style="list-style-type: none"> 8.1. Ensure the fume hood sash is pulled down as far as possible while still allowing free arm movement. 8.2. Tap on syringe to loosen any bubbles and depress plunger to leech solution into a paper towel to remove trapped air. 2 3 9. Attach needle tip to the syringe via the luer connection. 10. Load the syringe into the pump by inserting the needle tip through the hole in the plastic electrospinner housing and secure tightly within the pump with the black clamp (A). 4 <ol style="list-style-type: none"> 10.1. Ensure that the syringe flange is flush with the stationary pump block (B). 10.2. Bring the mobile pump block flush with the syringe plunger end by pressing its brass buttons in (C). 11. Turn the syringe pump on by pressing a switch behind the black clamp (A). <ol style="list-style-type: none"> 11.1. Press select twice and ensure volume is set to at least 3.5 ml; increase the number to at least 0.5 ml more than the total amount of solution in the syringe as needed. 11.2. Press select, scroll to Flow Rate and press select and ensure 5.5 ml/hr is inputted. 11.3. Press select, confirm that the screen displays a volume with an arrow. 12. Adjust slide and rotate settings on the controller box below the syringe pump to 3 and 6, respectively. 13. Attach the red, negatively charged wire to the needle tip <ol style="list-style-type: none"> 13.1. Ensure that the wire runs back through the legs of the syringe pump stand rather than along the walls of the enclosure. 5 14. Close the enclosure door and briefly engage the power supply by engaging the rightmost orange switch. 6 <ol style="list-style-type: none"> 14.1. Do not open the chamber or touch the needle, pump, pump stand, or any wires while the power supply is on. 14.2. Ensure that the switch in the center of the machine is set to negative polarity and the rightmost dial on the power supply is set to 12kV; turn the knob below to correct any deviation from this value. 14.3. Turn off the power supply.
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	<p>15. Press the Run/Stop button on the syringe pump to start.</p> <p style="text-align: center;"><u>B. Electrospinning Operation</u></p> <p>16. Wait for a bead to form on the tip of the syringe needle.</p> <p>16.1. Take note of the temperature, humidity, and volume ejected by the pump (on pump display) at the time of bead formation.</p> <p>17. Once the bead forms, turn on the slide/rotate switches and then the rightmost power supply switch. ⑦</p> <p>17.1. Take notes on Taylor Cone formation or any other unusual observations.</p> <p>18. Run the electrospinner until the syringe is empty, observing the process and taking notes along the way.</p> <p>18.1. The mandrel should become covered with PLGA and turn white soon after the electrospinning process starts.</p> <p>18.2. If the pump unit appears as though it will reach a displayed value of 3.5 ml before the syringe is empty increase the pump volume before the run is completed.</p> <p>19. Turn off the voltage supply, then the syringe pump, and finally the slide/rotate controls.</p> <p>20. Move the end block away from the syringe plunger, and remove the negatively charged wire from the needle tip.</p> <p>20.1. Open the enclosure and wipe any residual polymer solution from the needle tip before removing it through the hole in the chamber.</p> <p>21. Remove the syringe needle and dispose in the sharps container.</p> <p>22. Unload the mandrel from the electrospinner by unscrewing the distal end of the spinner and while taking care to only touch the mandrel pins.</p> <p>22.1. Place the scaffold on a clean paper towel until it can be placed inside the desiccator in 41-209.</p> <p>22.2. Note the time at which the scaffold was placed in the desiccator.</p> <p style="text-align: center;"><u>C. Cleaning the Electrospinner</u></p> <p>23. Thoroughly wipe down the entirety of the electrospinning collector and housing using paper towels and IPA.</p> <p>23.1. Take care when handling wires and wire connection points.</p> <p>23.2. Ensure no polymer coatings or webbings remain on the surface of the spinner or the inside of the enclosure.</p>	
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	<p>23.3. The collector may need to be removed from the fume hood to properly clean it; carefully unscrew and unplug the yellow cable from the control box, pass it through the enclosure, and unplug the ground wire from the collector before moving.</p> <p>24. Dispose of all vials, syringes (without needles), paper towels, polymer scraps, and contaminated gloves in the appropriate chlorinated waste stream container.</p> <p style="text-align: center;"><u>D. Scaffold Removal</u></p> <p>25. After desiccation for at least 24 hours, retrieve scaffold and remove the end sections with a scalpel blade.</p> <p>26. Gently twist the scaffold near the spring pin “t” until loosened, and continue this process down the entire length of the scaffold.</p> <p>27. While minimizing the pressure applied directly on the surface of the scaffold, slide the scaffold off the mandrel.</p> <p style="text-align: center;"><u>*Mandrel Polishing*</u></p> <p>Mandrels should be re-polished by a thorough abrading treatment with 4000 grit sandpaper every 3 months or as scaffold removal becomes more difficult.</p>	
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Revision History		
A	Toni Pipes	Fall 2013
B	Jakub Truty	Spring 2014
C	Evan Dowey	Summer 2017

APPENDIX I: SAMPLE CALCULATIONS WITH THE REVISED SOLUTION
MIXING PROTOCOL (APPENDIX G)

Example Calculation #1: 15wt.% PLGA in Chloroform

Known:

PLGA mass $M_p = 0.7835$ g

Polymer Concentration = 15wt. %

and so $M_t = (0.7835 \text{ g})/0.15 = 5.22$ g

Chloroform density $\rho = 1.49$ g/mL

Want:

Volume of Chloroform V

Calculations:

$$M_t = M_p + (V_1 * \rho_1) + \dots (V_n * \rho_n)$$

$$5.22 \text{ g} = 0.7835 \text{ g} + \left(V * 1.49 \frac{\text{g}}{\text{mL}} \right)$$

And so

$$M_s = V_1 * \rho_1 + \dots V_n * \rho_n$$

$$\frac{5.22 \text{ g} - 0.7835 \text{ g}}{1.49 \frac{\text{g}}{\text{mL}}} = V$$

$$\underline{2.978 \text{ mL} = V}$$

Example Calculation #1: 15wt.% PLGA in a 2:1 by volume Chloroform:Acetone Solution

Known:

PLGA mass $M_p = 0.7835$ g

Polymer Concentration = 15wt. %

and so $M_t = (0.7835 \text{ g})/0.15 = 5.22$ g

Chloroform density $\rho_1 = 1.49$ g/mL

Acetone density $\rho_2 = 0.784$ g/mL

2:1 solvent volume ratio = $V_1 = 2 * V_2$

Want:

Volume of Chloroform V_1

Volume of Acetone V_2

Calculations:

$$M_t = M_p + (V_1 * \rho_1) + \dots (V_n * \rho_n)$$

$$5.22 \text{ g} = 0.7835 \text{ g} + \left(V_1 * 1.49 \frac{\text{g}}{\text{mL}} \right) + (V_2 * 0.784 \frac{\text{g}}{\text{mL}})$$

And so

$$M_s = V_1 * \rho_1 + \dots V_n * \rho_n$$

$$5.22 \text{ g} - 0.7835 \text{ g} = V_1 * 1.49 \frac{\text{g}}{\text{mL}} + V_2 * 0.784 \frac{\text{g}}{\text{mL}}$$

And because $V_1 = 2 * V_2$ then

$$5.22 \text{ g} - 0.7835 \text{ g} = 2 * V_2 (1.49 \frac{\text{g}}{\text{mL}} + 0.784 \frac{\text{g}}{\text{mL}})$$

$$\frac{5.22 \text{ g} - 0.7835 \text{ g}}{2 * (1.49 \frac{\text{g}}{\text{mL}} + 0.784 \frac{\text{g}}{\text{mL}})} = V_2$$

And so $0.975 \text{ mL} = V_2$, $1.951 \text{ mL} = V_1$

APPENDIX J: ISO 7198:1998 KINK RADIUS TEST PROTOCOL

Taken directly from ISO 7198:1998

8.9 Determination of kink diameter/radius (A)

8.9.1 Principle

This test is intended to determine the radius of curvature required to begin “kinking” a vascular prosthesis.

8.9.2 Apparatus

Templates of radius ranging from 4 mm to 50 mm in increments of 1.5 mm are used. Alternatively, cylindrical mandrels of known diameter may be used

8.9.3 Sampling

Sampling shall be in accordance with clause 7.

8.9.4 Test procedure

The kink radius, to the nearest increment of the gauge, is determined before and during pressurization as appropriate.

Since kink radius may be affected by pressure, non-water-permeable prostheses should be tested at 100 mmHg internal pressure. Water at room temperature should be used unless kink behavior is affected by temperature. Water-permeable constructions may be tested at ambient pressure. The radius of the mandrel that first causes graft kinking is recorded.

Samples are placed in a radius template that does not cause kinking or narrowing. The template radius is decreased until slight narrowing or kinking of the prosthesis is determined.

Alternatively, a cylindrical mandrel may be used to determine kink radius. This is accomplished by forming a loop out of the test sample, and pulling the ends of the sample in opposite directions in order to reduce the loop until a kink is observed. The appropriate size cylindrical mandrel is placed within the loop to measure the kink radius.

8.9.5 Expression of results

The kink radius is measured in millimeters.

8.9.6 Test report and additional information

The test report shall include the mean and standard deviation of the kink radius of the sample prosthesis, the test conditions of temperature and pressure, and details required by 4.9.1.

Additional information, including the number of samples and the method of testing, shall be recorded together with the details required by 4.9.2.