FABRICATION OF INJECTABLE CELL CARRIERS
BASED ON POLYMER THIN FILM DEWETTING

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This dissertation describes the development of high aspect ratios of injectable cell carriers fabricated based on simple and cost-effective techniques utilizing dewetting of polymer thin films. The high aspect ratios of injectable cell carriers would be vital for tissue regeneration such as muscles, neurons, and blood vessels. Current injection based approaches have not truly mimicked the anisotropic structures. As results, in this study, two different injectable cell carriers have been developed to achieve these important structures.

Prior to generating the structures, the stability of poly(lactic-co-glycoic acid) (PLGA) films in common aqueous solutions to further utilize it for fabricating scaffolds. The results showed that, for relatively thinner films (< 30 nm), dewetting of PLGA films on Silicon-wafer under both DI-water and phosphate buffered saline (PBS) was mainly controlled by electrostatic interactions; for thicker films (> 30 nm), van der Waals interactions governed the instability. Under both BSA solution and culture medium, dewetting of PLGA films was suppressed and occurred only for a relative short period (≤ 24 h), and was found to be mainly controlled by electrostatic interactions. The suppression was caused by the changed surface properties (e.g., surface energy and zeta potential) of the polymer films due to protein adsorption.
The first construct was fibrous injectable cell/polymer micro-constructs. Patterned PLGA films were fabricated via featured polydimethylsiloxane (PDMS) stamps. The work focused on fibrous film generation by rupturing thin layers between thick patterns via dewetting. To maintain control over the continuous fibrous film generation, the imprinted films were first dewetted, and then placed on agarose-coated surfaces. As a result, cells seeded on the samples migrated to the PLGA surfaces during incubation time. A three days of incubation of cells on the bundle was found to be optimal, and using a tapered syringe barrel increased the injectability almost by two fold. More importantly, in vitro study using an alginate gel micro-environment showed a relatively high viability, ~78%, resulted for cells on the bundle after 7 days of incubation post injection, while the viability of free cells decreased to ~51% 7 days post injection (vs. 93% immediately after injection). Furthermore, injected bundles maintained at the injected locations whereas injected free cells were sparsely observed at the injected locations.

The second construct was tubular cell carriers generated by self-rolling bilayer films. Active and adhesive layers were consisted of PLGA and poly(D,L lactic acid) (PDLA) films, respectively. Initial strains were applied to the active layer, which folded when the PDLA layer detached on substrates by dewetting under cell culture conditions. Several types of tubes were obtained by initiating the rolling from different directions, which actuated depending on the aspect ratios of the rectangular patterns of bilayer films. After three days incubation of mouse embryonic fibroblasts (MEFs), rat smooth muscle cells (SMCs), and rat endothelial cells (ECs) on the bilayer films, it became tubes with encapsulation of cells.
DEDICATION

To my wife, Yun Kyoung, and my daughters, Jeena and Hannah. I greatly thank for your presence in my life.
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# TABLE OF CONTENTS

| LIST OF TABLES | xii |
| LIST OF FIGURES | xiii |

## CHAPTER

### I. INTRODUCTION

1.1 Importance of High Aspect Ratios of Injectable Cell Carriers .......... 1  
1.2 Objective of Research ................................................................. 3  
1.3 Overview of the Dissertation ....................................................... 4  

### II. BACKGROUND

2.1 Injectable Materials ........................................................................ 5  
2.2 Current Injection based Cell Therapy ............................................ 10  
2.2.1 Direct Injection of Free Cell Suspension ...................................... 10  
2.2.2 Hydrogel Systems ...................................................................... 13  
2.2.3 Non-Hydrogel based Systems ..................................................... 18  
2.2.4 Composite Systems ................................................................. 22  
2.2.5 Scaffold-Free Cell Delivery ....................................................... 26  
2.2.6 Other Potential Candidates for Injectable Scaffold Systems ......... 29  
2.2.7 Design Criteria for Generating Alternative Injectable Scaffolds ... 32  
2.3 Dewetting of Thin Polymer Films .................................................. 34  
2.3.1 Basics of Thin Polymer Dewetting ............................................ 34
2.3.2 Morphology in Dewetting Process

III. EXPERIMENTAL APPROACH

3.1 Materials

3.2 Dewetting of PLGA Thin Films under Aqueous Media

3.2.1 Modification of Si-wafer with PEG-Silane

3.2.2 Dewetting of PLGA Films

3.2.3 Surface Properties Determination

3.2.4 Zeta Potential Measurement

3.3 Fibrous Polymer Bundle Formation via Thin Polymer Film Dewetting

3.3.1 Surface Preparation

3.3.2 Flat PLGA Film Generation on Glass slides

3.3.3 PLGA Parallel Strips on Different Surfaces

3.3.4 Cell/PLGA Bundle Formation

3.4 Cell/Polymer Micro Bundles as a Noble Injection based Scaffold to Enhance Cell Viability and Retention

3.4.1 MTT Assay on Various Substrates

3.4.2 Feasibility of Bundle Injection

3.4.3 Preparation of Alginate Gel Environment

3.4.4 Viability and Proliferation of Cell/Polymer Bundle Injection

3.4.5 Live/dead Cell Staining

3.4.6 Retention Study using Alginate Gel in vitro Model

3.4.7 In vivo Assessment of Injected Cell/Polymer Micro Construct

3.5 Fully Biodegradable Injectable Tubes via Self-Rolling Bilayer Films for Cardiovascular Disease

3.5.1 Fabrication of Bilayer Films and Tubes Formation via Self-Rolling Bilayer Films
4.3.2 Morphological Observation and Injectability of Bundles ...................... 103
4.3.3 Effect of Geometries of Bundles on Injectability ............................... 106
4.3.4 Retention Study ................................................................................. 108
4.3.5 Viability Study .................................................................................... 112
4.3.6 Enhanced Viability of Post Injected Cells in vitro ............................... 117
4.3.7 Injectability of Cell/Polymer Fibrous Micro Constructs in vivo ........... 124
4.3.8 Summary ............................................................................................ 125
4.4 Fully Biodegradable Injectable Tubes via Self-Rolling Bilayer Films for Cardiovascular Disease ................................................................. 127
4.4.1 Introduction ....................................................................................... 127
4.4.2 Tube Formation via Self-rolling Bilayer Films in Cell Culture Condition ... 131
4.4.3 Initial Strain Effect on Tube Diameters and Estimation of Initial Strain during Film Generation Process ..................................................... 137
4.4.4 Capability of Cell Encapsulation in Tubes ........................................... 143
4.4.5 Aspect Ratio Effect on Tube Shapes ................................................... 147
4.4.6 Injection Feasibility of Tube ............................................................... 149
4.4.7 Inner Patterned Structures in Tube ...................................................... 151
4.4.8 Co-culture of SMCs and ECs in Bilayer Films .................................... 153
4.4.9 Summary ............................................................................................ 163
V. CONCLUDING REMARKS AND FUTURE STUDIES ............................ 164
5.1 Concluding Remarks ............................................................................... 164
5.2 Future Studies ...................................................................................... 167
REFERENCES .............................................................................................. 169
APPENDICES ............................................................................................... 188
APPENDIX A: DETERMINATION OF SURFACE TENSION AND ITS COMPONENTS OF AQUEOUS SOLUTIONS USING LIQUID-LIQUID INTERFACIAL TENSIONS .................................................. 189
<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Hydrogel based injectable scaffold.</td>
</tr>
<tr>
<td>2.2</td>
<td>Non-hydrogel based scaffolds.</td>
</tr>
<tr>
<td>2.3</td>
<td>Composite systems for injection based cell therapy.</td>
</tr>
<tr>
<td>2.4</td>
<td>Scaffold-free injection based cell therapy.</td>
</tr>
<tr>
<td>2.5</td>
<td>Common advantages and disadvantages of current injection based cell therapy.</td>
</tr>
<tr>
<td>4.1</td>
<td>The measured contact angles of DI-water, methylene iodide and ethylene glycol form on the PLGA film, Si-wafer and the PEG modified Si-wafer.</td>
</tr>
<tr>
<td>4.2</td>
<td>The measured interfacial tensions between the four aqueous solutions and three organic solvents: ethyl acetate, ethyl ether and benzaldehyde.</td>
</tr>
<tr>
<td>4.3</td>
<td>The surface energy and its components estimated based on the contact angle and interfacial tension values for the various materials used in this study.</td>
</tr>
<tr>
<td>4.4</td>
<td>Hamaker’s constants ($A_{MPS}$) of a PLGA film on Si-wafer and the PEG modified Si-wafer in aqueous solutions.</td>
</tr>
<tr>
<td>4.5</td>
<td>Zeta potentials ($\zeta$) of PLGA particles in aqueous solutions.</td>
</tr>
<tr>
<td>4.6</td>
<td>Values (contact angles of probe liquids) used for estimating surface energy and its components are summarized.</td>
</tr>
<tr>
<td>4.7</td>
<td>Contact angles of probe liquids on PDLA and its surface components.</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Alginate monomers: (a) β-D-mannuronate (M) and (b) α-L-guluronate (G), (c) Alginate acid</td>
</tr>
<tr>
<td>2.2</td>
<td>Representative synthetic biodegradable polymers; (a) poly(D,L-lactic acid), (b) poly(glycolic acid), and (c) poly(D,L-lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>2.3</td>
<td>Schematic of cell membrane disruption due to the extensional flow (not drawn to scale). Linear velocity of a fluid is significantly increased when the fluid from a large diameter (3.170 mm) of syringe enters to a small diameter (0.185 mm) of needle. The increased flow dynamics are large enough to disrupt the cell membrane. The figure is redrawn from ref. 51</td>
</tr>
<tr>
<td>2.4</td>
<td>Schematic of cryogelation. The figure is redrawn from ref. 62</td>
</tr>
<tr>
<td>2.5</td>
<td>Schematics of the fabrication of porous beads. (a) Gas forming, (b) W/O/W emulsion in a fluidic device. The figure is redrawn from ref. 75 and 76</td>
</tr>
<tr>
<td>2.6</td>
<td>Composite systems using electrospinning techniques. (a) Hyaluronan and methylcellulose blended hydrogels with electrospun genipin cross-linked collagen or a co-polymer of poly(ε-caprolactone-co-D,L-lactide) fibers. (b) Poly(ethylene glycol)-diacrylate hydrogel with electrospun poly(ε-caprolactone) fibers. The figures are redrawn from ref. 24 and 77</td>
</tr>
<tr>
<td>2.7</td>
<td>Spreading of B on a substrate of A (A: lost ΔAA; B: gained ΔAB; AB: gained ΔAAB)</td>
</tr>
<tr>
<td>2.8</td>
<td>Typical morphology changes of dewetting of thin polymer films</td>
</tr>
<tr>
<td>3.1</td>
<td>Mechanisms of chemical bonding with PDMS and a glass slide: 254 nm wavelength of UV creates free oxygen atoms and oxygen molecules. The process substitutes the non-polar groups (-CH₃) to polar groups (-OH). Chemical bonding proceeds by the joining of two surfaces. Strong chemical bonding process is occurred at a 100 °C of a hot plate</td>
</tr>
<tr>
<td>3.2</td>
<td>Procedures of fabrication of featured PDMS stamps and imprinted PLGA films on glass slides</td>
</tr>
<tr>
<td>3.3</td>
<td>Generation of fibrous PLGA sheets on agarose-coated surfaces</td>
</tr>
</tbody>
</table>
3.4 Fabrication of bilayer films. PDLA films were spin-coated and PLGA films were generated in between PDMS pads. Bilayer films were obtained by pressing the PLGA films on PDLA coated substrates. ................................................................. 56

3.5 Two types of injection for injectability study: (a) Conventional injection and (b) in-house design of injection. .............................................................60

3.6 Characterization of cell alignment on patterned surfaces ......................... 61

4.1 Time evolution of ~30 nm of PLGA films dewetting under various aqueous solutions. Films on Si-wafer in (a) water, (b) PBS, (c) cell culture medium, and (d) films on the PEG-modified Si-wafer in cell culture medium. Scale bar: 25 µm. .......................................................... 70

4.2 Time evolution of ~70 nm of PLGA films dewetting under various aqueous solutions. Films on Si-wafer in (a) water, (b) PBS, (c) cell culture medium, and (d) films on PEG modified Si–wafer in cell culture medium. Scale bar: 25 µm. ...72

4.3 Time evolution of (a) ~30 nm and (b) ~70 nm of PLGA films dewetting under 0.4 w/v % BSA in PBS. Scale bar: 25 µm. ................................................................. 73

4.4 Double-logarithmic plots of the maximum number of holes (N_h) as a function of PLGA film thickness (h) in aqueous solutions. For dewetting under (a) DI-water and PBS, the slopes of the best fit lines are −3.9 ± 0.3 (h: 10–100nm), −3.3 ± 0.2 (h < 30 nm), and −3.8 ± 0.2 (h > 30 nm) for DI-water and −4.5 ± 0.3 (h: 10–100 nm), −3.4 ± 0.2 (h < 30 nm), and −3.9 ± 0.2 (h > 30 nm) for PBS. (b) For dewetting under cell culture medium, the slopes of the best fit lines are −4.4 ± 0.6 (h: 10–100 nm), −3.2 ± 0.3 (h < 20 nm), and −2.7 ± 0.7 (h > 20 nm) for PLGA on Si-wafer and −5.1 ± 0.8 (h: 10–100 nm), −2.9 ± 0.1 (h < 20 nm), and −3.2 ± 0.4 (h > 20 nm) for PLGA on PEG-modified wafer. ................................................. 75

4.5 Two different routes of generation of fibrous cell/polymer constructs. Route 1: cells are seeded directly on imprinted PLGA films. During incubation, thin layers are dewetted and cells are migrated on the PLGA surfaces. Route 2: Fibrous films are generated by dewetting to remove thinner layers. Cells are seeded after transferring the fibrous PLGA sheets on substrates. ...................... 86

4.6 Proliferation of MEFs on different surfaces. (e) The absorbance of dissolved formazan formed during the MTT assay of cells attached on the four different surfaces. The optical images of cells attached on (b) flat PLGA films, (c) agarose-coated glass slides, and (d) PEG modified glass slides, (e) PLGA bundles placed on agarose coated glass slides. Statistical analysis by one-way ANOVA (n = 4, ***p < 0.001). Scale bar: 100 µm............................... 88

4.7 Generation of imprinted polymer films and different instabilities on several surfaces under cell culture condition. (a) Generation of PLGA imprinted films using a featured PDMS. Stabilities on different surfaces after seeding cells (MEFs, 5.0 × 10^4 cells/mL): (b) PDLA films on glass slides, (c) PLGA with
APTES on glass slides, (d) PLGA films on glass slides, and (e) PLGA films on PEG modified glass slides. Scale bar: 100 µm. ................................................................. 92

4.8 AFM scanned topographies of imprinted PLGA films and progress of dewetting of the films under 37 °C DI-water. (a) The imprinted film has parallel ridges (height: ~1 µm). (b) Thin layer between the ridges has a thickness of 43.7 nm, verified after dewetting. Progress of dewetting at the thin layers is shown in images (c) – (e) obtained by an optical microscope. (c) Holes are formed after 15 min. (d) Holes are combined to form rims after 30 min, and (e) the rims are incorporated to thick ridges after 90 min. (f) Sample stages are prepared by placing fibrous PLGA films on agarose coated glass slides (The size is 1.0 cm × 0.8 cm). Scale bar: 100 µm. ................................................................. 95

4.9 A route of producing cell/polymer bundles from individual polymer strips (a). Progress of bundles formation is shown in (b)-(e). Adjacent fibrous polymer strips are combined during incubation to form the bundles. Right after seeding cells (1 × 10^5 cells/mL) (b), the cells are randomly distributed; 4 h later (c), most of the cells attach on to polymer strips; d) 1 day after incubation, bundles are formed; and e) after 3 days incubation, the outer layer of each polymer bundle is fully covered with cells to form thick bundles (D ~ 80 µm). If strips are broken (arrow), they retracted and formed thicker bundles (D ~ 120 µm, f). Scale bar: 100 µm for (b)-(e) and 200 µm for (f). ................................................................. 97

4.10 The formation of cell/polymer fibrous micro-constructs with different cell types. (a) MEFs, 1 day of incubation, (b) iVPCs, 1 day of incubation, (c) iVPCs, 3 days of incubation, (d) human kidney 293A cells, 3 days of incubation, (e) human liver HepG2 cells, 3 days of incubation, and (f) rat ECs, 3 days of incubation. The seeding cell density was ~1 × 10^5 cells/mL. Scale bar: 100 µm. ................................................................. 98

4.11 Schematic illustration of bundle formation and subsequent bundle injection: (a) Cells are seeded on parallel strips of PLGA that is placed on a glass slide coated with a thin layer of agarose gel. (b) The cells proliferate on the polymer strips. (c) Bundles are sectioned into smaller pieces, and then (d) delivered into the target place by an injection with a G 20 needle. ................................................................. 102

4.12 Injectabilities of bundles are assessed using two different injection designs. (a) A conventional syringe/needle design, and (b) replacing the conventional syringe barrel with a tapered pipette tip. (c) The comparison of injectability of bundles with different incubation times using the two designs of injection. The images of bundles before (d) and after (e) injection for bundles formed after 1(1), 2 (2), and 3(3) days of incubation. The florescent images (far right) of the bundles with 3 days of incubation presented are also presented. As shown in the florescent images, cell death caused by injection is not significant. Significantly different groups are indicated by letter using two-way ANOVA, where “A” represents the highest mean (n = 30, p < 0.001). Scale bars: 100 µm. ................................................................. 104
4.13 Injectability of bundles with different aspect ratios. The percentage pass rate (~76 %) of bundles (3 days incubated) with a length of ~0.5 mm (b) and ~1 mm (c) are similar, but it is decreased (to ~60 %) using bundles with a length of ~2 mm (d). The diameter of the bundles are ~100 µm. Scale bar: 100 µm. Statistical analysis by ANOVA (n = 30; *p < 0.05).

4.14 Retention evaluation of cell/polymer bundle injected in vitro. (a) An in vitro environment made of sodium/calcium alginate gel containing injected cell/polymer bundles. (b) The dissolved state of the gel using 10 wt % sodium citrate after 7 days incubation. Enlarged images show bundles right after injection in the gel (c), and after 7 days incubation (d). Cell coverage on the bundles became thicker after 7 days of incubation (e2 and f2) as compared to its initial state (e1 and f1). The corresponding fluorescent images (e3 and f3) of (e2) and (f2) show that most cells are alive. Scale bar: 500 µm for (c) and (d), and 200 µm for (e) and (f).

4.15 Retention of dissociated cells in an alginate gel as an in vitro environment. Several bundles were also delivered in alginate gels as position markers to find the initially injected site. (a) A schematic representation of the locations imaged. (b) Right after injection and (c) after 7 days of incubation. The bundles were retained their initial locations (1). Individual cells became cell clusters at the top (2) and the middle (3) and no cells were found at the bottom. Near the bundles, individual cells (indicated by arrows in b5 and b6) could no longer be seen (c5 and c6) and they clustered and migrated to the bundles after incubation. Scale bar: 500 µm for (b1) and (c1), 200 µm for (b2–6) and (c2–6).

4.16 Cell viability before and immediately after injection, assessed by trypan blue. The viability of free cells (dark grey bars) before and immediately after injection was 97.6 ± 0.9 % and 92.8 ± 0.9 %, respectively. For bundles (lighter grey bars), the viability before injection was 88.0 ± 2.8 %, and it was 87.7 ± 2.2 % immediately after injection. Significantly different groups are indicated by letter using two-way ANOVA, where “A” represents the highest mean (p < 0.001).

4.17 Viability (a) and proliferation (b) of MEFs in an alginate gel environment for 14 days. Viability and proliferation data are obtained by counting live/dead cells with aid of a hemocytometer after staining the cells with trypan blue at each day. Significantly different groups are indicated by letter using two-way ANOVA, where “A” represents the highest mean (n = 3, p < 0.001)

4.18 Cell attachments on PLGA fibrous sheets at different locations during 7 days incubation in alginate gels: cells are initially attached on the polymer sheets (location 1) and cells are not initially attached on them (location 2). Scale bar: 100 µm.

4.19 (a) and (b) different focuses for representative images of cell attachment on polymer strips after dissolving an alginate gel. (c) Viability of free cell injection in an alginate gel with and without fibrous PLGA sheets in an alginate gel after 7
days incubation. Statistical analysis by one-way ANOVA (n = 3. ***p < 0.001). Scale bar: 100 μm................................. 120

4.20 Cell migration towards to the PLGA strips during cell/polymer bundle formation. (a) Randomly distributed cells right after seeding cells (5 × 10^4 cells/mL). (b) Enlarged image of (a). (c) Cell migration 30 min after seeding cells. Scale bar: 100 μm for (a) and 25 μm for (b) and (c). ........................................... 121

4.21 Cell migration from agarose surfaces to PLGA strips within the first hour after seeding the cells. Images were taken (a) right after seeding cells (5 × 10^4 cells/mL), (b) after 15 min, (c) 40 min, and (d) 1 h. Scale bar: 25 μm. .................... 123

4.22 Cell attachments on PLGA strips and no cell attachment on agarose surfaces after 4 h incubation (a). Its enlarged two boxed images of 1 for (b) and 2 for (c). Scale bar: 100 μm for (a) and 25 μm for (b) and (c). ........................................... 123

4.23 The representative images of injection results in vivo. Injection of (a) free iVPCs and (b) iVPCs/PLGA constructs in rat hearts four weeks after the injection therapy. (c) and (d) are the enlarged sections boxed in (a), (b) respectively. iVPCs were labeled with tdTomato (red) and the nuclei of other cells were stained by DAPI (blue). Scale bar: 100 μm. ........................................... 125

4.24 Schematic of cell encapsulation via self-rolling of bilayer films. ..................... 130

4.25 3-D tube formation via rolling of bilayer films. (a) Right after adding cell culture medium to the bilayer films (pattern 4 mm × 500 μm); (b) after 1 h, instabilities were seen in the films; (c) after 4 h, rolling from edges was visible; (d) after 12 h, most rolling occurred along (parallel to) the long sides; (e) after 48 h, tubes formed. (f) SEM images show the resulting tubes formed by rolling from two long edges of a bilayer. The thicknesses of bilayer films were 600 nm and 330 nm, respectively for PLGA and PDLS films. Scale bar: 400 μm for (a) – (d) and 100 μm for (e) – (f). ............................................................... 133

4.26 Comparison between (a) our approach and (b) hydrogel based self-rolling bilayer films. For both cases, the passive layers induce strain gradient on the active layers. In our cases, the passive layers also act as a temporal adhesive. .......... 134

4.27 The dependence of tube diameter on bending strain. The thicknesses of bilayer films were 600 nm and 330 nm, respectively for PLGA and PDLS films. a) After 12 h, films with a ε_x = 0 % were still rolling, while tube formation had been finished for all films with a ε_x of 2 to 5 %. b) After 48 h, the films with a ε_x = 0 % finally formed tubes, and the tubes resulted from films with a ε_x of 2 to 5 % reduced in their diameters. c) After 72 h, the diameters of all the tubes slightly reduced. Scale bar: 100 μm. ........................................................................ 140

4.28 The dependence of the tube diameter on bending strain at different time. The thicknesses of bilayer films were 600 nm and 330 nm, respectively for PLGA and PDLS films. Values represent mean and standard deviation (n = 20). .......... 141
4.29 The dependence of tube width on the thicknesses of bilayer films. Values represent mean and standard deviation ($n = 20$) .................................................. 142

4.30 Encapsulation feasibility in tubes using rat mouse fibroblasts at (a) 1-day, (b) 2-day, (c) 3-day, and (d) 7-day. Right images are bright field images and left images are florescent images after staining with CellTracker™ Green CMFDA. (e) Tube width changes during incubation time for 7 days. Statistical analysis by ANOVA ($n = 20; *p < 0.05$). Scale bar: 100 $\mu$m .................................................. 144

4.31 Viability of MEFs encapsulated in tubes for 7 days. Cells encapsulated in tube shows high viabilities during incubation time for 7 days compared to viabilities of cells seeded on agarose-coated surfaces. Significantly different groups are indicated by letter using two-way ANOVA, where “A” represents the highest mean ($n = 20, p < 0.05$) .................................................. 146

4.32 The rolling dependency of tube on aspect ratio of bilayer patterns with fixed width (500 $\mu$m) after seeding MEFs. (a) Diagonal rolling dominates on aspect ratio of 2:1. (a1) after 24 h, edges are still rolling. (a2) and (a3) After 48 h, tubes are done and the edges are perfectly rolled up. (b) Long-side and diagonal-rolling dominates on aspect ratio of 4.5:1. (b1) After 24 h, diagonal rolling to long side rolling are shown. Some films shows diagonal rolling from two different ends. (b2) After 48 h, tubes with long side rolling. (b3) tubes with diagonal rolling from two ends (arrows). (c) Two designs used for injectability. Scale bars: 100 $\mu$m for (a3) and others for 400 $\mu$m .................................................. 148

4.33 The dependency of injectabilities on design of injection and aspect ratio. Values represent mean and standard deviation ($n = 30$) .................................................. 150

4.34 The dependency of rolling direction on pattern direction (a1) perpendicular, (b1) parallel, and (c1) diagonal to long side of bilayer films with MEFs seeding. (a) diagonal direction of patterns results in long side rolling. The tub tube formation at (a2) 24 h and (a3) 48 h. (b) Parallel direction of patterns results in short side rolling. (b2) short side rolling at 12 h, and (b3) ribbon shape of final structure of bilayer films at 24h. (c1) Diagonal direction of patterns at initial state. Rolling starts from diagonal rolling for all aspect ratio of bilayer films: (c2) 4:1 and (c3) 8:1 at 4 h. (c4) After 12 h, long sides dominates on high aspect ratio and cells grow along with the direction of the inner patterns. (c5) 4:1 aspect ratio results in diagonal rolling (arrows) and (c6) results in long side rolling at 24 h incubation. 152

4.35 Encapsulation of (a) SMCs and (b) ECs and viability in tubes for 7 days. Bright field (left) and Floresent (right) images of SMCs stained with CellTracker™ Green CMFDA for live cells and Hoechst blue for nuclei encapsulated in tubes at (a1) 1 day, (a2) 2 days, and (a3) 3 days as well as those of ECs at (b1) 1 day, (b2) 2 days, and (b3) 3 days. .................................................. 155

4.36 Viabilities of (a) SMCs and (b) ECs on agarose (negative control) and in tubes from non-patterned bilayer films (pattern 500 $\mu$m × 4 mm). Significantly
different groups are indicated by letter using two-way ANOVA, where “A” represents the highest mean (n = 20, p < 0.001).

4.37 Inclination of SMCs depending on types of blood vessels: (a) large size blood vessels and (b) small size blood vessels.

4.38 Schematic of co-culture of ECs and SMCs.

4.39 SMCs alignment on patterned PLGA films. (a) SMCs on smooth PLGA films (b) Bright field image of SMCs alignment on parallel striped patterned PLGA films (spacing ~20 µm) on glass slides. (c) SMCs stained with CellTracker™ Green CMFDA are growth through the direction of the strips and (d) ECs stained with Hoechst blue are well attached on SMCs. SMCs (density: 1 × 10^5 cells/mL) were incubated for 20 h, and then ECs (1 × 10^5 cell/mL) were seeded on SMCs layer. Images were taken after 4 h incubation. Scale bar: 100 µm.

4.40 Pre-formed blood vessel generation. (a) 4 h after seeding SMCs on diagonal patterned bilayer films. ECs are seeded at this time. (b) After 24 h, tube formation is finished resulting in helical inner patterned tubes with (c) encapsulation of two types of cells: green florescent – SMCs and blue florescent – ECs. (d) After 48 h, tube formation maintains tube formation and e) two cells are well encapsulated inside of the tubes. (f) Confocal image of 3 days incubated tubes with encapsulated two types of cells. Scale bar: 500 µm for a) – (c) and 100 µm for (d) – (f).
CHAPTER I
INTRODUCTION

1.1 Importance of High Aspect Ratios of Injectable Cell Carriers

Although the injection of a high density cell suspension into injured organs is the easiest and fastest approach,\textsuperscript{1–7} its retention and viability at target sites are significantly low.\textsuperscript{8–12} As a result, polymeric scaffolds have been introduced to resolve such problems.\textsuperscript{13}

Polymeric scaffolds are used to provide temporal supports for cell attachment, proliferation, and differentiation to regenerate injured tissues.\textsuperscript{13,14} Precise delivery of the cell containing scaffolds to the target site and retained at the target site is an important issue. Currently, delivery of cells with the scaffolds can be achieved by two different ways: injection and implantation.\textsuperscript{13} Injection-based therapy has been widely studied since it does not need surgery, is minimally invasive, reduces patient’s discomforts, and thereby, is a simple and cost-effective method compared to implantation. Significant research has conducted in this regard to develop injectable cell carriers. The most common injectable scaffolds include (1) hydrogel-based porous matrix, (2) non-hydrogel based scaffold and (3) composites of hydrogel and additives. Tissue engineering expects that irregular shapes of defects can be filled with those scaffolds incorporated with cells, and the cells are formed to a living tissue structurally and functionally similar to that
found in nature as the scaffolds gradually degrade.\textsuperscript{13,15} Not to be distinguishable for adequate function with the host organ, the scaffolds should have similar mechanical and structural properties with the extracellular matrix. However, these approaches have not truly mimicked native tissue matrix might be due to its material and processing limitations. For example, hydrogels cannot induce directional growth of cells, since its soft nature does not allow nano and micro-sized topographies that are found in native tissues.\textsuperscript{16,17} Non-hydrogel based scaffolds are generally fabricated by an oil-water emulsion technique and the final structures are microspherical shapes,\textsuperscript{18–20} which are not relevant to native tissues. Considering surface topographies induce directional growth,\textsuperscript{21} which determines function of tissues, incorporating fibrous and high aspect ratios of scaffold design is a prerequisite to truly mimic native tissues such as neurons, muscles, and blood vessels.

Among current approaches, electrospinning techniques have been adopted to achieve fibrous structures; however, the final products are normally 2-dimensional mats, which are not injectable.\textsuperscript{22,23} To generate injectable scaffolds by utilizing the electrospinning technique, several approaches have been introduced, including sonication\textsuperscript{24} and cryogenic grinding\textsuperscript{25} to produce fibrous structures by scission. However, the electrospun fibers cannot be injectable, thus mainly are blended with hydrogels. As a result, these sectioned fibers share the same limitations as those of hydrogels (e.g., diffusion limitations and direct cell-to-host organ contact). Therefore, the development of alternative approaches is imperative.
1.2 Objective of Research

Key aspects of high aspect ratios of injectable cell carriers include inducing directional cell growth using its topographies and providing structure integrity to promise cell retention and survival in vivo. Although immobilization of functional biomaterials on polymeric surfaces is an important issue to increase initial attachment and later tissue functions, this work focuses on the design of high aspect ratios of cell/polymer micro-constructs by utilizing commercially available biodegradable PLGA and PDLA and evaluation of their potentials as injectable cell carriers. Several approaches have been introduced for generating polymer-based fibers and tubular structures with high aspect ratios. However, the development of simple and inexpensive lab-scale techniques is still valuable.

The primal objective of this work is to develop simple, cost-effective, and noble techniques to generate injectable scaffolds with high aspect ratios and to evaluate to the viability and retention of cells injected with these scaffolds. Two types of scaffolds have been developed in the study. First, the scaffold is composed of loosely packed fibrous PLGA strips. The fibrous strips were generated by dewetting of patterned PLGA films. Cells and the fibrous strips self-assembled cell/polymer micro-constructs during incubation. This resulted in cells that were grown in and out side of the constructs. In vitro and in vivo injection evaluations of cells grown on these fibrous strips showed that they could be potential injectable cell carriers. Second, the scaffold is in three-dimensional (3-D) tubular formation via self-rolling bilayer films. In this work, an initial pre-determined strain was applied on the PLGA film (i.e., active layer), which was placed
on the PDLA film (i.e., adhesive layer). The stress relaxation from the initial strains applied on the PLGA films resulted in continuous folding until the strains were completely released. The scaffolds were examined for encapsulation of several types of cells and they were further examined as possible injectable pre-formed blood vessels.

1.3 Overview of the Dissertation

The outline of this dissertation is as follows. In this chapter, the importance of the high aspect ratios of injectable cell carriers and the main objective of this work are briefly presented. Chapter II provides background information in current injectable scaffolds for cell delivery and fundamentals for dewetting phenomenon. Chapter III will present materials, experimental procedures, and characterization methods. Chapter IV describes the main results and discussions of the study. Chapter V concludes this study along with the advantages and disadvantages of the two micro carriers and possible future studies possible future directions of this research.
CHAPTER II
BACKGROUND

In this background chapter, the current injectable scaffolds for cell delivery will be presented, along with some fundamentals for dewetting phenomenon. Active research has been carrying out for injection based cell therapy. In section 2.1, the materials used for injection will be briefly summarized. The progress and issues of current approaches will be discussed in Sec. 2.2. In Sec. 2.3, scaffold design reported but not examined as injectable scaffolds will be shown as a potential injectable scaffold. In Sec. 2.4, some requirements for an alternative injectable scaffold will be summarized to overcome problems of current approaches. In Sec 2.5, fundamentals for dewetting phenomenon will be provided.

2.1 Injectable Materials

Currently, hydrogels have been mostly utilized for fabrication of injectable scaffolds. Hydrogels are 3-dimensional (3-D) hydrophilic, cross-linked polymeric networks that can absorb a significant amount of water or biological fluids. The cross-linking can be achieved physically or chemically, and the gelation kinetics and the subsequent properties of hydrogels can be controlled using numerous cross-linking reactions. Injectable hydrogels can be obtained either from natural or synthetic polymers.
Natural derived polymers are frequently selected, since these hydrogels either include components of the extracellular matrix (ECM, e.g. collagen, fibronectin, and fibrinogen) or present a chemical structure similar to natural glycosaminoglycans (GAGs, e.g. alginate, hyaluronic acid, chitosan), offering an intrinsic advantage over synthetic hydrogels.\textsuperscript{30} Many studies have shown that these nature materials provide favorable microenvironments for cells.\textsuperscript{31,32} Among such hydrogels, alginate hydrogels are one of the most used injectable materials, since they form hydrogel using non-toxic solvents (e.g. CaCl\textsubscript{2}). The G units of alginate, composed of $\beta$-D-mannuronate (M subunit) and $\alpha$-L-guluronate (G subunit), are cross-linked together in groups of four by multivalent cations, such as Ca\textsuperscript{2+} (Figure 2.1).\textsuperscript{33–35} It has tunable Young’s modulus ranging from 1 to 100 kPa and shear modulus ranging from 0.02 to 40 kPa.\textsuperscript{36} In addition, they can be further modified to improve their mechanical and biochemical properties to better mimic the native ECMs. For example, arginine-glycine-aspartic acid (RGD) sequence can be functionalized with hydrogels via carbodiimide chemistry in the case of grafting with alginate.\textsuperscript{37} For a better modification to mimic the natural ECM, protease-labile crosslinking peptide (proline-valine-glycine-leucine-isoleucine-glycine, PVGLIG) that is cleaved by metalloproteinase (MMP) can be grafted.\textsuperscript{38,39} Some general concerns for using natural materials include restricted control over their physic-chemical properties, inability to moderate their degradation rates, challenges in sterilization and purification techniques, and also pathogen/viral issues when obtaining from different sources.\textsuperscript{40}
Figure 2.1 Alginate monomers: (a) β-D-mannuronate (M) and (b) α-L-guluronate (G), (c) Alginate acid.
For synthetic biomaterials, poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and poly(D,L-Lactic-co-glycolic acid) (PLGA), their respective copolymer, have been extensively used as synthetic 3-D scaffold biomaterials (Figure 2.2). Necessary criteria such as biocompatibility, processability, and controlled degradation are fulfilled with these polyesters.\(^{41}\) These biomaterials degrade hydrolytically via mass erosion and the glycolic/lactic acid byproducts are physiologically removed through metabolic pathways. Their molecular weight, ratio of copolymers, and polydispersity can be easily tuned to adjust the degradation rate. In addition, standard processes to prepare a wide variety of 3-D scaffolds using those materials have been well established (e.g. salt leaching, sintering, porogen melting, and nano-fiber electrospinning).\(^ {42-45} \) One of major concerns for utilizing synthetic polymers includes their poor inherent bioactivity, e.g., poly(ethylene glycol), PEG. Therefore, the critical point for improving synthetic polymers is to modify them with biological or chemical compounds to improve their cellular response. Synthetic injectable hydrogels can be made to contain growth factors (e.g. vascular endothelial growth factor and platelet-derived growth factors) that are released at the repair site for the length of time necessary to create an environment conducive to tissue regeneration.\(^ {46} \) Since the growth factors have short half-life, timing and optimal amounts could be considered for retaining their bioactivity and effective releasing at the target site. To enhance cell adhesion, RGD-containing sequences into the injectable scaffold is incorporated to promote cell specific function.\(^ {47} \) Another serious concern for utilizing polyesters in bio-applications is inflammation in vivo due to the decrease of local pH by increased acidic byproducts when the polyesters degrade. However, other researchers have a different opinion that such concern is exaggerated.
since body fluid can dilute to maintain normal pH level.\textsuperscript{48,49} For example, Chang et al. measured the local pH after injection of a block copolymer hydrogel (PEG and PLGA) underneath of subcutaneous tissue. The initial pH was as low as 4, however, it recovered to neutral pH after 10 h.

![Representative synthetic biodegradable polymers](image)

Figure 2.2 Representative synthetic biodegradable polymers; (a) poly(D,L-lactic acid), (b) poly(glycolic acid), and (c) poly(D,L-lactic-co-glycolic acid).
2.2 Current Injection based Cell Therapy

Currently, several types of injection based cell therapy have been introduced. In this chapter, the current systems are categorized to injectable systems of hydrogels, non-hydrogels, biodegradable polymer/hydrogel composites, and scaffold-free cell sheets/clusters. Their approaches and progress will be discussed.

2.2.1 Direct Injection of Free Cell Suspension

In an early stage of cell therapy, a majority of studies had been focused on direct injection of cell suspension, since it could be conducted without surgery and minimally invasive. A variety of cell types had been delivered in this manner, including myoblast, neural stem cell, hepatocytes, bone marrow-derived cells, and ES cells.\(^1\)\(^-\)\(^7\) These studies have shown that the cells delivered by direct injection can repair damaged tissue. Specifically, Mangi et al. showed that genetically enhanced mesenchymal stem cells can repair infarcted myocardium.\(^6\) They used a retroviral vector to overexpress the prosurvival gene Akt in mesenchymal stem cells (MSCs) before implantation in ischemic myocardium (rats). The Akt protein overexpression was greatly enhance MSCs survival and prevent pathologic remodeling after infarction, with impressive improvement in cardiac output. Capone et al. verified that neurosphere-derived cells reduced functional impairment and neuronal damage due to the ischemia and reperfusion injury.\(^7\)

In order to enhance tissue regeneration in cell therapy, many studies have pointed out that at least two major requirements should be accomplished, regardless of cell types and targeted tissues. First, transplanted cells should be homogeneously distributed in the host tissue, since the regeneration could be proceeded via secretion of trophic factors
from the cells, not only direct participated cells in regeneration of the tissue.\textsuperscript{12} Second, initial retention of transplanted cells should be high, since long term engraftment rates are directly related to initial retention of the transplanted cells.\textsuperscript{8-10} However, none of such requirements could be achieved via direct injection and many studies have continuously reported its limitations.\textsuperscript{11,12,50,51} For example, Malliaras et al. revealed that long term engraftment rates were poor since the majority of delivered cells die or were washed away within the first week.\textsuperscript{12} Not only that, Hou et al. showed that free cell injection failed to deliver and retain cells at the desired site.\textsuperscript{50}

Besides the above mentioned issues associated with host organs, Aguado et al. revealed that the majority of cell death was caused by stretching forces on cells due to extensional flow during injection.\textsuperscript{51} According to the authors, cells can experience three types of mechanical forces that could lead to cell disruption during injection of cell suspension through a syringe needle (G 28). First, a pressure drops across the cell. Second, shearing forces due to linear shear flow, and last, stretching forces due to extensional flow (Figure 2.3).\textsuperscript{51}

To summarize, direct injection of cell suspension is simple and time-effective method. However, it has critical issues not only at the host organ, but also during injection. Major reasons of low retention of injected free cells are likely the massive leaking of cells out of the injected site during admiration and no structure integrities of dissociated cells that can retain at the host tissues. For these reasons, utilizing biomaterial carriers became popular as a mean for onsite retention and long-term survival.
for injection based cell therapy. It is also supported by the study of Stabenfeldt et al., which emphasized that the use of an appropriate biomaterial carrier to provide successful clinical outcome.\textsuperscript{11} The following sections, various approaches to enhance the initial retention of injected cells will be discussed with respect to design of scaffold design.

Figure 2.3 Schematic of cell membrane disruption due to the extensional flow (not drawn to scale). Linear velocity of a fluid is significantly increased when the fluid from a large diameter (3.170 mm) of syringe enters to a small diameter (0.185 mm) of needle. The increased flow dynamics are large enough to disrupt the cell membrane. The figure is redrawn from ref. 51.
2.2.2 Hydrogel Systems

Early studies in tissue engineering scaffolds were focused on hydrogels with naturally derived materials such as alginate, fibrin, and gelation\textsuperscript{52,53}. Since they showed a lack of mechanical properties (e.g. modulus), synthetic polymers have become more widely utilized. Physical hydrogels that are temperature dependent on sol-gel phase transition behavior have been attractive due to their injectability as sol state before/during injection and gel state after injection upon body temperature. The representative polymers are poly(ethylene oxide)-\textit{block}-poly(propylene oxide)-\textit{block}-poly(ethylene oxide) (PEO-PPO-PEO, Pluronic), PLGA-PEO-PLGA tri-block copolymers, and poly(N-isopropylacrylamide, NIPAAm).\textsuperscript{54–57} Among the sol-gel hydrogels, Pluronic has been widely utilized to deliver cells for cartilage regeneration.\textsuperscript{54} Pluronic gels with a concentration of 20 % (w/v) have succeeded to produce cartilage.\textsuperscript{58} However, the gels immediately leaked out at the injected site due to the dilution by the body fluid (i.e., Pluronic normally becomes a solution at a concentration of < 20 % (w/v)), a similar problem as that of free cell injection. To enhance the proper stability \textit{in vivo}, PEG-g-PLGA and PLGA-g-PEG were prepared by grafting of poly(lactic-co-glycolic acid), PLGA, and poly(ethylene glycol).\textsuperscript{59} The polymers are consisted of hydrophobic core (PLGA) and hydrophilic shell (PEG) and the authors believed that the gelation is achieved by aggregation of the micelles. By blending of two polymers with different rates, controllable degradation time (1 week to 3 month) of the gel can be achieved.

Another usage of hydrogels is to protect cells during injection. Aguado et al. showed that shear-thin delivery of hydrogels could be an attractive method for protecting
cells during injection. According to their results, cells delivered with the cross-linked alginate hydrogel (G’ = 29.6 Pa) displayed a viability of ~90 % after injection, which is significantly improved comparing to that (~60 %) of injection of a free cell suspension in Newtonian solutions (i.e., buffer) and 1 % of non-cross-linked alginate solution in phosphate buffered saline (PBS). According to their results, the higher viability was mainly due to the mechanical gelation of the alginate gel, which protected cell membrane from the extensional flow caused during injection. Similarly, Yan et al. also confirmed the protection of cells by the shear-thin approach (β-hairpin peptide-based hydrogel) via catheter injection (250 μm-ID capillary). In their study, they quantified shear-thin effect on flow behavior of the hydrogels with encapsulation of MG 63 cells. The hydrogel and cells experienced plug flow.

For better mimicking the native tissue, one of the most important design criteria is to mimic the native tissue, i.e., fibrous structures of extracellular matrix, nanofibrous physical gel has been developed using decellularized native tissue (e.g., heart). In their work, ventricular or pericardial tissue was harvested and decellularized. It was then sectioned, lyophilized, and milled to create a fine powder. The powder was solubilized using pepsin, and they confirmed the presence of several extracellular matrix components such as collagen and glycosaminoglycan (GAG) content. The gel solution was a viscous liquid at room temperature and became a gel at 37 °C. The gel was composed of a nano-fibrous and meso-porous structure (by SEM). The gel precursor was injected into the myocardium by a 27 gauge Myostar catheter (a rodent model). After 11 days, the
migration and infiltration of endothelial cells and smooth muscle cells were observed as well as arteriole formation.

Unless physical gel system showed some clinical progress, there are some concerns. First, when cell-aqueous hydrogel precursor solution is injected, in situ gelation of hydrogel might cause harmful effect on encapsulated cells. Second, in situ forming gels have problem in controlling its regular pore size and homogeneous pore distribution within the gel due to the dilution with body fluid. Third, transporting of oxygen, nutrients, and secreted products from cells originated from bulky structures after administration might cause a massive cell death. Lastly, viscous hydrogel precursor might not be able to protect cell membranes from disruption during injection, since the precursor has no desired structure integrity as Aguado et al. verified.51

To avoid these issues associated with a physical gel system, more recently, cryogel systems have been introduced.62 In this work, a pre-formed scaffold using alginate was generated by cryogelation (Figure 2.4). During the process, unfrozen/semi-frozen phases restrict the reactants, which form a cross-linked network by polymerization. Thereby, pores are generated from the ice crystals nucleated from the aqueous phase by acting as porogens. By melting these ice crystals above the freezing temperature (-20 °C), interconnected macroporous networks can be obtained. After generating the cryogels, cells incorporated by infiltration. Since cryogel consists of sufficient structural integrity and strength, the gel protects encapsulated cells during injection (shear-thin delivery) and recovered to its initially designed shape after injection. One of the major drawbacks is its
non-physiological processing condition of cryogelation. Therefore, cells were incorporated by infiltration after gels were prepared. The authors mentioned that the pore size sometimes was not large enough for the infiltration; however, the methods of controlling regular size and interconnectivity of pores were not provided.

As mentioned above, hydrogels have shown encapsulation and protection effectiveness, resulting in long-term retention at host organ compared to injected free cells. However, it has some intrinsic problems needed to be solved. One of the major concerns is the diffusion problem, i.e. transportation of nutrients and secretions for cells are limited. In addition, the gels would interrupt direct contact of encapsulated cells with the host organ, which can reduce long term cell viability and low incorporation of injected cells to the host organ.63,64

Figure 2.4 Schematic of cryogelation. The figure is redrawn from ref. 62.
Table 2.1 Hydrogel based injectable scaffold.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Gelation</th>
<th>Cell types</th>
<th>Applications</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO-PPO-PEO</td>
<td>Physical gel</td>
<td>Pheochromocytoma cells</td>
<td>Drug delivery/Tissue engineering</td>
<td>52</td>
</tr>
<tr>
<td>p(NIPAAm-co-AAc)/RGD</td>
<td></td>
<td>-</td>
<td>Nerve regeneration</td>
<td>53</td>
</tr>
<tr>
<td>Pluronic F-127</td>
<td></td>
<td>Chondrocytes</td>
<td>Cartilage repair</td>
<td>56</td>
</tr>
<tr>
<td>PLGA-g-PEG</td>
<td></td>
<td>Chondrocytes</td>
<td>Cartilage repair</td>
<td>57</td>
</tr>
<tr>
<td>Decellularized myocardial matrix</td>
<td></td>
<td>Endothelial/smooth muscle cells</td>
<td>Cardiac tissue engineering</td>
<td>59</td>
</tr>
<tr>
<td>Alginate</td>
<td>Shear-thin delivery</td>
<td>-</td>
<td>N/S</td>
<td>49</td>
</tr>
<tr>
<td>β-hairpin peptide-based hydrogel</td>
<td></td>
<td>-</td>
<td>N/S</td>
<td>58</td>
</tr>
<tr>
<td>Fibrin</td>
<td>-</td>
<td>Chondrocytes</td>
<td>Cartilage repair</td>
<td>50</td>
</tr>
<tr>
<td>Alginate/peptide (PVGLIG)</td>
<td>Casting (3-D disc caster)</td>
<td>Mesenchymal stem cells</td>
<td>N/S</td>
<td>38, 39</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Oil/water emulsion</td>
<td>Adipose derived stromal cells</td>
<td>Adipogenesis</td>
<td>51</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Cryogelation</td>
<td>NIH 3T3 fibroblast</td>
<td>N/S</td>
<td>60</td>
</tr>
<tr>
<td>Gelatin/RGD</td>
<td></td>
<td>NIH 3T3 fibroblast</td>
<td>N/S</td>
<td>91</td>
</tr>
<tr>
<td>PEDGA</td>
<td></td>
<td>Mesenchymal stem cells</td>
<td>N/S</td>
<td>90</td>
</tr>
</tbody>
</table>

Note: N/S; Not specified.
2.2.3 Non-Hydrogel based Systems

Since physically cross-linked hydrogels normally lack of the desired mechanical properties (e.g. low modulus), synthetic polymers were also actively researched. A significant number of researches and reviews have reported the processing and use of cell microcarriers based on synthetic biocompatible/biodegradable/bioabsorbable polymers (e.g. PLGA), natural materials (gelatin), or their combinations, for different tissue regenerations including bone, cartilage, dermal, hepatic, and adipose.

Microspheres with a size ranging from hundred nanometers to micrometer have been commonly used as injectable carriers for cell delivery. Initially, non-porous PLGA microspheres were utilized as a microcarrier in vitro and in vivo. For example, chondrocytes incubated with PLGA microspheres (diameter: 63 – 199 µm) produced cartilage tissue similar to native tissue compared to that of free cell injection. In addition, they confirmed that cells migrated and packed into the spaces where the PLGA microspheres degraded.

In spite of its several positive effects, non-porous microspheres have been considered to be an inefficient carrier system due to the large mass ratio of materials to cells attached on their surfaces. Also, it is hard to control the morphologies of exterior and interior of the microspheres using conventional microsphere fabrication techniques including emulsification and emulsion polymerization. To enhance cell-loading capacity on microspheres, highly porous microspheres were produced by a gas foaming method using ammonium bicarbonate as a gas foaming agent (Figure 2.5a). Huang et al.
adopted this gas foaming approach in combination with a microfluidic device to produce porous microspheres with a controllable pore size (Figure 2.5b). The prepared microspheres needed to be incubated with cells in a spinner flask for 12h, and then each microsphere was individually transferred to a well of the 96-well plate to inhibit the aggregation of microsphere with other microspheres for the cells to reached the confluent level. The resulted PLGA porous beads have a diameter of ∼250 µm with a pour size of ∼50 µm. The pores were interconnected and large enough for cells to infiltrate to achieve a high cell loading. They believed that the large pores allow effective mass transfer of oxygen and nutrients to cells considering that the diffusion limitation of oxygen in dense cellular structures is ∼200 µm. The authors also confirmed promising results that the delivery of human amniotic fluid stem cells (hAFSCs) incubated with the microspheres improved regional contractile capacity of a rat heart by neovascularization and myocardial regeneration. However, there are two major concerns for this work. First, incorporation of cells into the microspheres, prior to injection, was time consuming. According to the authors, 500 beads were normally needed for a rat model (250 – 300 g). In addition, the surface of the scaffold is smooth, which does not truly mimic the fibrous structures of native tissue.

For better mimicking of the native tissue using microspheres, a novel technique has been recently developed to fabricate injectable nano-fibrous (NF) hollow microsphere. In this work, star-shaped poly(lactic acid), (SS-PLLA) was synthesized using poly(amidoamine) dendrimers as initiators. The NF hollow microspheres were generated by emulsifying the polymer solution (in tetrahydrofuran) in glycerol with
rigorous stirring, and fibrous structures were formed through phase separation of the mixture by quenching in liquid nitrogen. Solvent extraction and freeze-drying were then applied to obtain highly porous hollow microspheres with open holes on the NF spheres without any templates. In this work, the microspheres were investigated as injectable scaffolds for cartilage regeneration \textit{in vivo}. The scaffolds showed the regeneration of cartilage to exhibit the similar structure as the native cartilage. The fibrous structures, which resembled extra cellular matrix of native tissues, were still too fine for cells to infiltrate. It had one or two large pores where cells were expected to migrate inside of the spheres during cell seeding using a spinal flask.

Figure 2.5 Schematics of the fabrication of porous beads. (a) Gas forming. (b) W/O/W emulsion in a fluidic device. The figure is redrawn from ref. 75 and 76.
Table 2.2 Non-hydrogel based scaffolds.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Shapes</th>
<th>Fabrication Technique</th>
<th>Culturing Method</th>
<th>Application</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA-g-poly(L-lysine)</td>
<td>Sphere</td>
<td>Oil/water emulsion</td>
<td>Spinner flask</td>
<td>Cartilage repair</td>
<td>71</td>
</tr>
<tr>
<td>PLGA</td>
<td>Sphere</td>
<td></td>
<td>Shaker</td>
<td>Cartilage repair</td>
<td>18</td>
</tr>
<tr>
<td>ppAAM-treated PLGA</td>
<td>Sphere/crushed particle</td>
<td></td>
<td></td>
<td>Brain repair</td>
<td>87</td>
</tr>
<tr>
<td>PLGA</td>
<td></td>
<td></td>
<td></td>
<td>Cartilage repair</td>
<td>89</td>
</tr>
<tr>
<td>PLGA</td>
<td>Sphere</td>
<td>Gas forming</td>
<td>Infiltration</td>
<td>N/S</td>
<td>75</td>
</tr>
<tr>
<td>PLGA/neuregulin-1 HA-PLGA</td>
<td>Sphere</td>
<td>Oil/water emulsion</td>
<td>Drop wise</td>
<td>Cardiac infarction</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bone repair</td>
<td>86</td>
</tr>
<tr>
<td>Star shaped PLLA</td>
<td>Nanofibrous sphere</td>
<td>Self-assembled</td>
<td>Drop wise / infiltration</td>
<td>Cartilage repair</td>
<td>72</td>
</tr>
<tr>
<td>PLGA</td>
<td>Porous bead</td>
<td>Microfluidic device</td>
<td>Infiltration</td>
<td>Cardiac infarction</td>
<td>76</td>
</tr>
</tbody>
</table>
2.2.4 Composite Systems

The composite systems basically attempt to increase the mechanical strength of hydrogels, which are considered as mechanically weak materials. For example, Kang et al. verified that compounding of hydrogel with PLGA particles increases the mechanical strength compared to hydrogel alone. The combination showed the increased storage modulus and viability of the cells injected in vitro. Some other studies adopted composite systems by incorporating electrospun fibers and hydrogels, which not only increased the mechanical strength, but also enhanced the functions of the scaffolds. Two recent examples were compared below.

Hsieh et al. investigated the combination of electrospun fibers with hyaluronan hydrogels and hyaluronan-methylcellulose blended hydrogels (Figure 2.6a). Genipin crosslinked collagen and a copolymer of poly(e-caprolactone-co-DL-lactide) (PCL-co-DLLA) were electrospun to prepare fibers. The collected fiber mats were sonicated until it became smaller sized fragments, and the generated fragments were blended with hydrogels (5 mg/mL). The authors verified the composite’s injectability using a G30 needle. They further examined its potential as an injectable scaffold for neural stem/progenitor cells in treating spinal cord injury. Cells migrated and aggregated as clusters around the fibers. In this study, notable difference was found as compared to their controlled free cells in gels without fibers. Cell aggregation in hydrogels without fibers was more significant than that in the hydrogel/fiber combination after 7 days incubation. In the latter case, cells were more sparsely dispersed. They further assessed gene expression using gels without and with fibers. Interestingly, cells incubated in gels
contained fibers showed much higher quantities of genes (βIII-tubulin, 2',3’-cyclic nucleotide 3’-phosphodiesterase, and glial fibrillary acidic protein) compared to cells incubated in gels alone. For two types of fibers (genipin cross-linked collagen and PCL-co-DLLA) in gels, the combination of PCL-co-DLLA and gels showed higher proliferation and differentiation compared to genipin cross-linked collagen. However, the authors did not clearly explain this result and did not include cytotoxicity data. Another drawback of this approach is the control of the size and shape of the fibers during the sonication step to generate fibers from electrospun mats. Therefore, uniform shape and size of fibers by sonication would need more study to elucidate the advantage of this scaffold. Furthermore, the feasibility of the scaffold should be further examined in vivo, since the scaffold is mainly composed of a physical hydrogel, which has issues described above.

Coburn et al. have developed a similar approach. They fabricated injectable three dimensional porous fiber/hydrogel composites, which were prepared using electrospun fibers that were not fragmented (Figure 2.6b). Different from previous electrospinning technique that polymer fibers were collected on water or a solid surface to produce a dense fiber structure with only a few millimeters thick, they generated a 3-D fibrous mesh with similar porosity as that of the hydrogel. PCL was electrospun on the surface of an ethanol/water solution (ratio 9/1), which was used to reduce surface tension, and the spun fibers penetrated through the solution, resulting in a loosely packed fibrous 3-D structure. The scaffolds were frozen and vacuum dried. Cell infiltration was achieved by simple mixing of a cell suspension in the poly(ethylene glycol) diacrylate,
PEGDA, hydrogel solution containing the fibers, which then solidified by photosynthesis to form the 3-D fibrous structure. The authors believed that this 3-D fiber/hydrogel truly mimics the native tissues, but without providing any direct evidences. By using human mesenchymal stem cells, they observed cell morphology in the composite gel and the gel alone. While stellate morphologies and long extension of cells were observed in the composite gel, the cells inside the pure gels were rounded. Coburn et al. also commented that injection of the 3-D scaffold was easy; however, the information (dimensions and types) of the syringe used and the injection results (injectability, cell viability before and after injection) were not reported.

![Figure 2.6 Composite systems using electrospinning techniques.](image)

(a) Hyaluronan and methylcellulose blended hydrogels with electrospun genipin cross-linked collagen or a co-polymer of poly(ε-caprolactone-co-D,L-lactide) fibers. (b) Poly(ethylene glycol)-diacrylate hydrogel with electrospun poly(ε-caprolactone) fibers. The figures are redrawn from ref. 24 and 77.
Table 2.3 Composite systems for injection based cell therapy.

<table>
<thead>
<tr>
<th>Hydrogels/Additives</th>
<th>Fabrication of Additives</th>
<th>Cell Types</th>
<th>Applications</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAMC/p(CL:DLLA)</td>
<td>Electro-spinning</td>
<td>Multi-potent neural stem/progenitor cells</td>
<td>Spinal cord injury</td>
<td>24</td>
</tr>
<tr>
<td>PEG/PCL</td>
<td></td>
<td>Mesenchymal stem cells</td>
<td>Cartilage repair</td>
<td>77</td>
</tr>
<tr>
<td>Chitosan hydrogel/PLGA particle</td>
<td>Oil/water emulsion</td>
<td>Chondrocytes</td>
<td>Chondrogenesis</td>
<td>65</td>
</tr>
</tbody>
</table>
2.2.5 Scaffold-Free Cell Delivery

So far, cells have been cultured either on a two-dimensional or in a three-dimensional environment prior to injection. Su et al. insisted that 2D culture was considered to be non-physiological and could lead to changes in gene or protein expression in cells. On the other hand, 3-D culture in cell medium condition allows 3-D aggregates up to a few hundred microns by self-assemble through cell-cell interactions with minimal resistance to better maintain their stemness. To examine their claim, the authors firstly generated 3-D spheres of human urinary bladder papilloma cell line RT4 and human embryonic kidney epithelial cell line HEK293 cells on low attachment dish coated with agarose. Their results demonstrated that these cells were reprogrammed and acquired stemness.

However, it is reported that high ratio of cells were dead at the core of such cell clusters due to the diffusion issue. An earlier study found that neurospheres with a diameter of 220 μm showed viable cells only at the periphery of the neurospheres. Other studies also verified that dense cellular structures develop hypoxia at exceeding maximum oxygen diffusion distance, typically about 150 ~ 250μm. Regular production of cell sphere smaller than this thickness would be increased with this technique. Nevertheless, they did not show how to control regular sizes of the 3-D spheres with any data about the sizes of 3-D spheres as well as injectability with respect to their sizes.
In fact, biomimetic injectable cell delivery system has been developed using thermoresponsive polymers. In this approach, cells were seeded and proliferated on the thermoresponsive polymer surfaces until it reached a confluent level. The surfaces of polymers are hydrophobic surface above its lower critical solution temperature (LCST) (typically ~32 °C), but switched to hydrophilic below its LCST, resulting in detachment of the cell sheets from the surfaces. This technique was initially developed to apply peripheral injury as a patch or construct complex tissue implants by stacking several cell sheets. In different way using this technique, several studies have adopted this technique for fabricating injectable cell fragments or cell body. Chen et al. examined the injectability of cell sheet fragments in cardiac disease. Cell sheets were generated on thermoresponsive hydrogel prepared by pouring aqueous methylcellulose on TCPS, which is aqueous state at 20 °C and gelled at 37 °C. The cell sheets were fragmented with regular sizes (380 µm × 380 µm) using a stainless screen to allow clear injection, and detached by decreasing temperature ~20 °C. The authors examined cell sheet fragment injection in infarcted myocardium of rat and pigs, and observed that they were entrapped in the muscular tissues at the site injected. Compared to the dissociated cell injection, it increased significant vascular density and improved the function of an infarcted heart. They further developed this technique for generating cell bodies using a multi-welled methylcellulose hydrogel system using a 96-well plate. Since diameters of injectable drug carriers were reported 60 ~ 250 µm, which provide physical structures could maintain in the interstitial tissue, they generated cell bodies with a diameter ~195 µm. The authors claimed that the cell bodies were injectable, however, the injectability and viability before and after injection were not provided in this paper. Although the lack
of feasibility in vitro, results of in vivo study using infracted rat heart showed significantly enhanced cell retention at short term (day 1) and engraftment with host organ at long term (week 4).

Table 2.4 Scaffold-free injection based cell therapy.

<table>
<thead>
<tr>
<th>Substrate materials</th>
<th>Fabrication technique</th>
<th>Shape</th>
<th>Cells</th>
<th>Applications</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNIPAAm</td>
<td>Cell sheet engineering</td>
<td>Cell sheet (10 × 10 cm)</td>
<td>Mesenchymal stem cells</td>
<td>Bone repair</td>
<td>90</td>
</tr>
<tr>
<td>Methyl-cellulose</td>
<td>Cell sheet fragments</td>
<td></td>
<td></td>
<td>Intramuscular administration</td>
<td>83</td>
</tr>
<tr>
<td>Methyl-cellulose</td>
<td>Cell bodies (sphere)</td>
<td></td>
<td>Human amniotic fluid stem cells</td>
<td>Myocardial infarction</td>
<td>84</td>
</tr>
<tr>
<td>-</td>
<td>Floating culture</td>
<td>Sphere</td>
<td>Neural stem cells</td>
<td>Nerve generation</td>
<td>79</td>
</tr>
</tbody>
</table>
2.2.6 Other Potential Candidates for Injectable Scaffold Systems

In this section, two types of tube structures currently available but not examined as injectable carriers will be discussed with respect to its advantageous and disadvantageous.

2.2.6.1 Tube Generation using a Sucrose Core Template

As previously reported by others, directional growth of cells guided by nano- or micro-tracks generated either chemically or topographically has shown to be a useful tool for regulating cell function. Lim et al., showed that controlling scale and pattern in chemical and topographic substrate patterning can enhance specific cell regulating cues for biomedical applications. In addition, numerous studies have demonstrated that the direction of cell growth can determine tissue functions, especially, for muscle and nervous tissues. Many studies have shown that micro- or nano-structures can also decide the proliferation or differentiation of cells. Since current injectable scaffolds typically do not provide such tracks to guide growth of cells, it is worth to discuss about such types of scaffolds currently available but not specified as an injectable system for their potential use as injectable scaffolds.

Li et al., introduced hollow fiber bundles composed of each fibers with high aspect ratios. By melt spinning technique with caramelized sucrose, core template was generated, which could then be encapsulated with a thin layer of biodegradable PLLA by dip-coating the core in the PLLA solution (in chloroform). Hollow fiber tubes were generated by dissolving the sucrose core in an aqueous solution (e.g., cell medium).
Tubes with a wall thickness ranging from 0.75 to 3 \( \mu m \) and an external diameter ranging from 8 to 100 \( \mu m \) could be easily generated by varying the PLLA concentration. Since the tubular scaffold was generated first, cell loading was achieved by pipetting through the edges of the tube. The cell loading is not time-efficient and easy to handle. The wall of the tubular scaffold can be collapsed or broken since the tube is needed to be held with aid of forceps. Moreover it is difficult when it is cut into smaller sizes for applying as injectable scaffolds.

2.2.6.2 Tube Formation via Self-Rolling Bilayer Films

The use of self-rolling films are able to form 3-D structures without templates, which is different from the traditional template-based approaches that particles or fibers are utilized as templates and hollow structures are obtained after the removal of the core. Inorganic\(^{109}\) and polymer-based bilayers\(^{110,111}\) are folded due to the relaxation of internal stresses obtained from intrinsically different properties of the two layers, such as lattice mismatch, thermal expansion mismatch, or swellability mismatch. Polymer-based self-folding films could be formed into a tube by swellability of the active layer (e.g. hydrogel-based polymer) while the passive layer inhibits expansion (e.g. polycaprolactone).\(^{111-113}\) Such approach is promising for biological applications (e.g. cell encapsulation) since the active layer can be swelled by cell culture medium during incubation. More importantly, the rolling of a rectangular bilayer could be actuated in three different scenarios: long-side, short-side, and diagonal rolling depending on the aspect ratio or the thickness ratio of the two layers (active and passive layer).\(^{114}\) As a result, different shapes of the tube can be obtained. Tube structure via self-rolling of bi-
layer films have been actively studied leading by the Ionov group. In 2010, Zakharchenko et al., introduced partially biodegradable self-rolling tubes utilizing polycaprolactone (passive layer) and poly(NIPAM-ABP), copolymer of PNIPAM and 4-acryloylbenzophenone.\textsuperscript{103} In this work, they demonstrated the encapsulation of microparticles in the tube during rolling by lowering the temperature below the LCST (28 °C) of poly(NIPAM-ABP) and released them by increasing temperature to 37 °C. In 2011, Zakharchenko et al., created tubular scaffold by utilizing fully degradable polymers (active layer: Polysuccinimide, passive layer: Polycaprolactone).\textsuperscript{112} This fully biodegradable scaffold was demonstrated to be a promising candidate for cell delivery by using yeast. One of the disadvantages of this work is that the rolling of polysuccinimide-based bilayers is only determined by the kinetics of hydrolysis. Therefore rolling might be hard to control. In 2014, Stroganov et al., developed more biocompatible tubular scaffold utilizing gelation (active layer) and polycaprolactone (passive layer).\textsuperscript{113} They demonstrated that neural stem cells could be encapsulated in the rolled-up tubes during their formation and survival for a considerable period of time. One of the drawbacks on their work is the use of two thick layers. To achieve tube with a diameter around \( \sim 10 \, \mu \text{m} \), 100 nm thick polycaprolactone and 1.7 \( \mu \text{m} \) thick gelatin layer were needed. The diameter can be increased by increasing the thicknesses of two layers, and therefore the larger diameter could need much thicker layers, which can limit the diffusion of nutrients for cells and interrupt cell-cell connection. In addition, for fast degradation of the tubular scaffold, polycaprolactone is not a good choice as materials, since it needs over 2 years to degrade.
2.2.7 Design Criteria for Generating Alternative Injectable Scaffolds

As discussed, current approaches have shown issues related to low retention, low viability for long term \textit{in vivo} and low grafting with host organ (Table 2.5). Their advantages and disadvantages based on earlier sections are summarized in Table 2.5. Therefore, development of an alternative injectable scaffold is required to meet the shortcomings of current approaches.

First, the scaffold should promote directional growth. Second, for proper cell survival and growth, the scaffold should not exhibit problems corresponding to the transport of nutrients and metabolites to and from the scaffold. Therefore, scaffold should be smaller than 250 \textmu{}m. Third, the scaffold should protect cells during injection. Fourth, the scaffold should pose the suitable mechanical and physical properties to protect cells and increase the retention of cells at the target site. Lastly, the scaffold should allow direct contact of cells with the host organ.
Table 2.5 Common advantages and disadvantages of current injection based cell therapy.

<table>
<thead>
<tr>
<th>Injectable system</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell suspension</td>
<td>- The simplest and most minimally invasive.</td>
<td>- Low retention at host organ</td>
<td>1–7, 11,12, 50,51</td>
</tr>
<tr>
<td></td>
<td>- Fast preparation time.</td>
<td>- No protection of cells during injection.</td>
<td></td>
</tr>
<tr>
<td>Hydrogels</td>
<td>- Easy cell encapsulation.</td>
<td>- Low cell-to-host organ contact.</td>
<td>51,52, 54,58, 61–64</td>
</tr>
<tr>
<td></td>
<td>- Irregular shapes of defects can be filled.</td>
<td>- Limited diffusion of nutrients and oxygen for cells.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Biomaterials can be loaded with cells.</td>
<td>- Initial mechanical properties can be altered due to the dilution with body fluid.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Gels can be prepared before and after injection in vivo.</td>
<td>- Low mechanical properties.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Directional growth of cells is not possible.</td>
<td></td>
</tr>
<tr>
<td>Non-hydrogels</td>
<td>- Well established process to generate uniform size of carriers (e.g. water-oil emulsion method).</td>
<td>- Most of the shapes are solid microspheres, which are different shapes of native tissue.</td>
<td>18–20, 65,70, 71–76,</td>
</tr>
<tr>
<td></td>
<td>- Direct contact of cell-to-host organ.</td>
<td>- Large mass ratio of materials to cells attached on their surfaces.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Fibrous networking or surface modification methods are available to enhance proliferation and survival of cells in vivo.</td>
<td>- Microsphere aggregation during cell culture.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Directional growth of cells is not possible (microspheres).</td>
<td></td>
</tr>
<tr>
<td>Polymer/hydrogel Composites</td>
<td>- Mechanically strong hydrogel based scaffold can be generated.</td>
<td>- Low cell-to-host organ contact.</td>
<td>24,65, 66,77</td>
</tr>
<tr>
<td></td>
<td>- Directional growth of cells can be induced.</td>
<td>- Limited diffusion of biomaterials for cells.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- For physical hydrogels, leaking out or lose their mechanical after injection.</td>
<td></td>
</tr>
<tr>
<td>Scaffold free cell sheet/clusters</td>
<td>- Do not need scaffold fabrication.</td>
<td>- Cutting process causes cell death where blades apply.</td>
<td>79,83, 84,90</td>
</tr>
<tr>
<td></td>
<td>- Direct cell-to-host organ contact is achieved.</td>
<td>- For cell bodies, maximum oxygen diffusion distance (~250 µm) need to be considered to eliminate cell death at the core.</td>
<td></td>
</tr>
</tbody>
</table>
2.3 Dewetting of Thin Polymer Films

In this section, basics of thin polymer dewetting and its morphological evolutions will be described.

2.3.1 Basics of Thin Polymer Dewetting

Dewetting is a well-known phenomenon and occurs in thin polymer films when they are heated above their glass transition temperature ($T_g$). In our incubation system, cells grow until they are aligned and proliferated on the patterned polymer films at 37 °C, the films would dewet if the polymer has a lower $T_g$ than 37 °C or not dewet if the polymer has a $T_g$ of more than 37 °C. The polymers PLGA and PDLA used in this project have 37 °C and 55 °C $T_g$ respectively. As reported earlier in water, polymers could be swollen by water and $T_g$ could be decreased more 10 °C for PLGA and PDLA. Therefore, under cell culture conditions (37 °C), dewetting of those polymers is expected.

Dewetting can be estimated by calculating a spreading coefficient for a system.

As shown in Figure 2.7, if B spreads on A, areal changes for the system are:

![Figure 2.7: Spreading of B on a substrate of A (A: lost $\Delta A_A$; B: gained $\Delta A_B$; AB: gained $\Delta A_{AB}$).](image)

Figure 2.7 Spreading of B on a substrate of A (A: lost $\Delta A_A$; B: gained $\Delta A_B$; AB: gained $\Delta A_{AB}$).
The differential form of Gibbs free energy change for the system is:

\[ dG = \left( \frac{dG}{dA_A} \right) dA_A + \left( \frac{dG}{dA_B} \right) dA_B + \left( \frac{dG}{dA_{AB}} \right) dA_{AB} \]  \hspace{1cm} (1)

or rewrite,

\[ dG = \gamma_A dA_A + \gamma_B dA_B + \gamma_{AB} dA_{AB} \]  \hspace{1cm} (2)

where, \( dG < 0 \), spontaneous spreading occurs and \( dG > 0 \), no spreading occurs.

We may define the spreading coefficient as,

\[ S_{B/A} = -\left( \frac{dG}{dA_B} \right) \]  \hspace{1cm} (4)

Therefore we have,

\[ S_{B/A} = \gamma_A - \gamma_B - \gamma_{AB} \]  \hspace{1cm} (5)

\( S_{B/A} \) is positive if spreading is accompanied by a decrease in free energy and is negative if dewetting is predicted to occur.

In the case that the polymer film is immersed in water, the spreading coefficient is rewritten as below,

\[ S_{M(P/S)} = \gamma_{SM} - \gamma_{PM} - \gamma_{SP} \]  \hspace{1cm} (6)

where, P: Polymer, S: Substrate, and M: Water

From the Young-Dupre equation,

\[ \gamma_{SM} = \gamma_{SV} + \gamma_{MV} - W_{SM} \]  \hspace{1cm} (7)

\[ \gamma_{PM} = \gamma_{PV} + \gamma_{MV} - W_{PM} \]  \hspace{1cm} (8)

\[ \gamma_{SP} = \gamma_{SV} + \gamma_{PV} - W_{SP} \]  \hspace{1cm} (9)

where, V: Air
By applying the geometric means of work of adhesion of different surface energy components developed by Good & van Oss, $S_{M(P/S)}$ became:

$$S_{M(P/S)} = -2\gamma_p - 2 \left( \sqrt{\gamma_S^{LW} \gamma_M^{LW}} + \sqrt{\gamma_S^+ \gamma_M^-} + \sqrt{\gamma_S^- \gamma_M^+} \right)$$

$$+ 2 \left( \sqrt{\gamma_P^{LW} \gamma_M^{LW}} + \sqrt{\gamma_P^+ \gamma_M^-} + \sqrt{\gamma_P^- \gamma_M^+} \right)$$

$$+ 2 \left( \sqrt{\gamma_S^{LW} \gamma_P^{LW}} + \sqrt{\gamma_S^+ \gamma_P^-} + \sqrt{\gamma_S^- \gamma_P^+} \right)$$

(10)

2.3.2 Morphology in Dewetting Process

The dewetting process of thin polymer films has unique evolution steps in respect to morphology (Figure 2.8). Under the right conditions ($> T_g$ and $S_{B/A} < 0$), instabilities caused from a substrate and surrounding evolve into ruptures on the free surface of the film. The ruptures grow towards the substrate, and after reaching the substrate, they form holes, exposing the substrate to water. After the hole opening, the holes grow laterally and combine with neighbor holes to form polygon rims and decay of the rims into droplets due to Rayleigh instability (capillary brake up) of the surface of long cylindrical masses, resulting from the tendency to reduce surface area of the polymer.

![Figure 2.8 Typical morphology changes of dewetting of thin polymer films.](image)
CHAPTER III
EXPERIMENTAL APPROACH

In this chapter, the experimental procedures associated with fundamental study of
dewetting thin polymer films under aqueous solutions and two scaffolding techniques are
described in detail, including sample preparation, process, and characterization. Mouse
embryonic stem cells were chosen as a model cell to load on the micro-constructs and
later several other types of cells were utilized for the potential use of biological
applications.

3.1 Materials

Sulfuric acid (98 % H$_2$SO$_4$), H$_2$O$_2$ (30 % technical grade), HPLC-grade toluene,
and ethyl acetate were purchased from Fisher Scientific; poly(lactic acid-co-glycolic
acid) (PLGA, 50:50, $M_w$ = 30 000 – 60 000), bovine serum albumin (BSA), and
phosphate buffered saline (PBS), sodium alginate, sodium citrate, trypan blue, and
fluorescein diacetate, propidium iodide were were from Sigma-Aldrich; 2-
[Methoxypoly(ethyleneoxy)propyl]trimethoxysilane (CH$_3$)(CH$_2$CH$_2$O)$_{6-9}$(CH$_2$)$_3$
Si(OCH$_3$)$_3$, PEG-silane) was purchased from Gelest Inc.; 3-aminopropytriethoxysilane
(APTES); Poly(D,L-lactic acid) PDLA was from PolysciencesInc; polydimethylsiloxane
(PDMS, Sylgard® 184) was from Dow Corning; agarose (UltraPur™ agarose) was from
Life Technologies; 6 and 24 well culture plates were from USA Scientific; mouse
embryonic fibroblast was from Millipore; rat induced vascular progenitor cells (iVPCs) were donated from Northeast Ohio Medical University (NEOMED); human embryonic kidney 293A cell line, human liver cell line (HepG2) were from ATCC; rat endothelial cells (ECs) and rat smooth muscle cells (SMCs) were from Cell Applications; 0.45 nm PTFE filters were purchased from GE Water & Process Technologies; 1 mL of syringe and G 20 needles (D: 584 µm) were from Becton, Dickson and Company; cell culture medium contained 85 v % DMEM cell medium and 15 v % fetal bovine serum, both from Invitrogen; Si-wafer was purchased from Silicon Quest International, Inc. and glass microscope slides were from Fisher Scientific.

3.2 Dewetting of PLGA Thin Films under Aqueous Media

In this part, detailed procedures of surface preparation, dewetting of PLGA thin films under aqueous media, and characterization will be described. Surfaces are Si-wafer wafer and PEG modified Si-wafer. Aqueous solutions are Di-water, PBS, 0.4 wt % BSA solution, and DMEM cell culture medium.

3.2.1 Modification of Si-wafer with PEG-Silane

A previous procedure was followed for the modification.120 Briefly, Si-wafers (cut to 1 cm × 1 cm pieces) were cleaned and oxidized, and then placed in a PEG-silane solution (3 mM of PEG in toluene with 0.8 mL of HCL per liter of solution). The modification process was allowed to occur for 18 h at room temperature. After the modification, the surfaces were thoroughly rinsed with toluene and sonicated for 2 min each in toluene, ethanol, and deionized water, and then dried by blowing a stream of N₂.
gas. The PEG-silane modified Si-wafers were characterized using contact angle measurements (advancing and receding) of three different probe liquids: water, ethylene glycol and methylene iodine.

3.2.2 Dewetting of PLGA Films

Thoroughly cleaned Si-wafers or PEG-silane modified Si-wafers were spin-coated, at a spin speed of 2000 rpm, with different concentrations (0.25 to 2.5 wt %) of PLGA solutions in ethyl acetate. After drying of ethyl acetate from the spin-coated film inside a vacuum (< 100 mTorr) oven at room temperature overnight, the film thickness was measured using an ellipsometer. Each of the samples was placed inside a well of a 24 well plate, and then 1 mL of the liquid, pre-heated to 37 °C, was carefully added to each well. Dewetting of PLGA films with a thickness of 10 to 100 nm in DI-water, PBS, BSA/PBS, and cell culture medium, was carried out inside a CO₂ incubator at 37 °C. The morphologies of samples at different stages of dewetting were observed using an optical microscope (IX70, Olympus). To obtain better images, some samples were removed from the aqueous solution, and those from PBS, BSA/PBS and culture medium were carefully dipped into a large DI-water bath (> 200 mL) to remove inorganic/organic materials that might complicate the final morphology of the dewetted samples, and then dried gently with a stream of N₂ prior to imaging. At least 10 different locations at each film were randomly imaged (0.169 mm²) to count number of holes (N_H) by eyes and it was presented with mean ± standard deviation (SD) after converting to 1 mm² scale.
3.2.3 Surface Properties Determination

Surface energy and its components of each of the solid surfaces (PLGA, Si-wafer, and PEG-silane modified Si-wafer) were estimated from the contact angles of water, ethylene glycol, and methylene iodine formed on the solid surface. The contact angles were measured with the aid of the Ramé–Hart Contact Angle Goniometer. The surface energy of substrates was determined using the van Oss–Chaudhury–Good approach incorporated in the Young–Dupré equation or equation (11)

\[(1 + \cos \theta)\gamma_L = 2\left(\sqrt{\gamma_S^{\text{LW}}\gamma_L^{\text{LW}}} + \sqrt{\gamma_S^+\gamma_L^-} + \sqrt{\gamma_S^-\gamma_L^+}\right)\]  

where, \(\theta\) is the contact angle of a liquid formed on a solid surface, \(\gamma\) refers to the surface tension or surface energy of a liquid or a solid, subscripts L and S denote the probe liquid and the solid substrate, respectively, the superscript LW symbolizes the Lifshitz van der Waals component, and superscript + and – denote the electron accepting and electron donating parameter, respectively.

Surface tension (\(\gamma\)) and its components of water, PBS, 0.4 w/v % BSA in PBS, and cell culture medium were determined using liquid–liquid interfacial tensions of the test liquid and three probe liquids with known surface tension components (\(\gamma^{\text{LW}}\) and the acid-base component \(\gamma^{\text{AB}}\)) and acid-base parameters (\(\gamma^+\) and \(\gamma^-\)). The interfacial tensions were measured using the pendant drop method. To estimate the values, the van Oss–Chaudhury–Good approach was utilized, which related the interfacial tension (\(\gamma_{ij}\)) to surface tensions (\(\gamma_i, \gamma_j\)) and their components and parameters as:
\[
\gamma_{ij} = \gamma_i + \gamma_j - 2 \left( \sqrt[4]{\gamma_i^{LW} \gamma_j^{LW}} + \sqrt{\gamma_i^+ \gamma_j^-} + \sqrt{\gamma_i^- \gamma_j^+} \right)
\]

\[
= \left( \sqrt[4]{\gamma_i^{LW}} - \sqrt[4]{\gamma_j^{LW}} \right)^2 + \gamma_i^{AB} + \gamma_j^{AB} - 2 \left( \sqrt{\gamma_i^+ \gamma_j^-} + \sqrt{\gamma_i^- \gamma_j^+} \right)
\]

(12)

When a mono-polar liquid (i) containing only \( \gamma_j^- \) was used as the probe liquid, equation (12) can be simplified to,

\[
\gamma_{ij} = \left( \sqrt[4]{\gamma_i^{LW}} - \sqrt[4]{\gamma_j^{LW}} \right)^2 + \gamma_i^{AB} - 2 \sqrt{\gamma_i^- \gamma_j^+}
\]

(13)

The values of \( \gamma_i^{LW}, \gamma_j^{AB}, \) and \( \gamma_j^+ \) in equation (13) can then be solved using interfacial tensions of the test liquid (j) and 3 mono-polar liquids (i.e. i). In our study, ethyl acetate, ethyl ether, and benzaldehyde were chosen as the mono-polar probe organic liquids. ~5 mL of each of the three solvents was placed in a quartz spectrometer cuvette, and an aqueous pendant drop was formed inside the organic solvent. To avoid the evaporation of the liquids, the opening of the cell was covered with a paraffin tape. The interfacial tension was determined using the expression below,

\[
\gamma_{ij} = \frac{\Delta \rho g De^2}{H}
\]

(14)

where, \( g \) is the gravitational acceleration, \( De \) is the equatorial diameter of the drop, and \( H \) is the shape factor, which is a function of \( Ds/De \), with \( Ds \) being the diameter measured at a distance \( De \) up from the bottom of the drop. The value of \( H \) can be obtained from empirical relationships. More than 30 drops of each aqueous solution/organic solvent pair were examined to determine the interfacial tensions.
3.2.4 Zeta Potential Measurement

0.3 wt % of PLGA in ethyl acetate was spin-coated on 1 cm × 1 cm of cleaned glass slides. After drying of ethyl acetate for 2 h in a fume hood, the films were dewetted at 80 °C inside a water bath for 20 sec, at which time, the films had completely dewetted into droplets that left on the slide. The samples were sonicated in water or PBS at room temperature for 5 min to remove the droplets from the slides into water or PBS. To adsorb proteins on the droplets, dewetted samples were incubated in BSA/PBS or cell culture medium at 37 °C inside a CO₂ incubator for 12 h. The samples were removed and rinsed with PBS, and then sonicated in PBS for 5 min to collect droplets. Zeta potential of PLGA droplets suspended in water or PBS and that of protein adsorbed PLGA droplets suspended in PBS were measured using dynamic scattering.

3.3 Fibrous Polymer Bundle Formation via Thin Polymer Film Dewetting

Fabrication of fibrous PLGA films by dewetting is described in this part. The detailed procedures are included preparation of various surfaces, fabrication of parallel strips on PLGA films using a featured PDMS stamp, and cell/polymer bundle formation. Detailed characterizations are also described.

3.3.1 Surface Preparation

Prior to modifying or film generation on glass slides (Fisher Scientific, 1cm × 1cm), the pieces were cleaned using a freshly prepared piranha solution [30 % H₂O₂ (Fisher Scientific) and 70/30 (v/v) of 98 % H₂SO₄ (Fisher Scientific)] followed by rinsing with DI water purified in house (with a conductivity of 0.1 mS or less). A previous
procedure was followed for the PEG modification on the cleaned glass slide. Briefly, the cleaned glass slides were oxidized, and then placed in a PEG-silane solution (3mM of PEG in HPLC-grade toluene (Fisher Scientific) with 0.8 mL of HCl per liter of solution). The modification process was allowed to occur for 18 h at room temperature. After the modification, the surfaces were thoroughly rinsed with toluene and sonicated for 2 minutes each in toluene, ethanol, and deionized water, and then dried by blowing a stream of N₂ gas.

3.3.2 Flat PLGA Film Generation on Glass slides

To prepare PLGA flat surface on glass slides, 3.5 wt % of PLGA solution in HPLC-grade ethyl acetate (Fisher Scientific) was passed through a micro-filter (Acrodisc 13 mm syringe filter with 0.45 µm nylon membrane, Gelman) to remove any impurities. Then 100 µL of the solution was spin coated (2000 RPM for 30 s) on the cleaned glass slides, which resulted 196 ± 14 nm thick films (3 different locations were measured by elipsometer and averaged from 3 samples) to avoid dewetting of PLGA films during incubation time. The surfaces stayed in a fume hood for 2 h and baked at 50 ºC in a vacuum oven (< 100 mTorr) to remove residual organic solvent for overnight.

3.3.3 PLGA Parallel Strips on Different Surfaces

In this work, we utilized featured PDMS (Sylgard 184, Dow Corning) stams to fabricate parallel strips of PLGA films on different surfaces including PEG-modified or un-modified glass slides and agarose-coated surfaces. We first fabricated the featured PDMS stamps via Fracture-induced structuring method developed by our group.
Briefly, flat silicone sheet (1.5 cm × 1 cm, thickness: 0.5 mm) and SiO\textsubscript{x} substrates (2 cm × 1.5 cm) were oxidized for 6 min by UV/ozone to form a silica layer on top of the silicone sheet while also cleaning the glass surface. The silicone sheet pressed onto the glass with the silica layer at the interface, with both substrates being chemically bonded together (Figure 3.1). After annealing at 100 °C of heat for 40 min to enhance the reaction, peeling of the bonded silicone/glass substrates at the interface induces parallel fractures that provide the round shape patterns on the silicone, and triangular shape patterns of silicone on glass surface. ∼20 µm spacing between strips were generated using these techniques. For distributing even pressure during imprinting process, a piece of glass slide (the same size of the PDMS stamp) was attached on the backside of the patterned PDMS stamp.

5 wt % PLGA solution in HPLC-grade acetone was passed through a microfilter and 20 µL of 5 wt % PLGA, (50:50, \( M_w: 35,000 \sim 60,000 \), Sigma-Aldrich) solution in HPLC-grade acetone (Fisher Scientific) was imprinted using the featured PDMS (Figure 3.2). The stamp was pressed with the finger pressure for 2 min to solidify the polymer. After releasing the stamp carefully, the films on substrates were dried overnight at a 45 °C vacuum oven (< 100 mTorr) to remove residual solvent.
Figure 3.1 Mechanisms of chemical bonding with PDMS and a glass slide: 254 nm wavelength of UV creates free oxygen atoms and oxygen molecules. The process substitutes the non-polar groups (-CH$_3$) to polar groups (-OH). Chemical bonding proceeds by the joining of two surfaces. Strong chemical bonding process is occurred at a 100 °C of a hot plate.
Figure 3.2 Procedures of fabrication of featured PDMS stamps and imprinted PLGA films on glass slides.
To prepare PLGA parallel strips on agarose-coated surfaces, 100 µL of 1 wt % agarose solution (~80 °C) in water was spread on the cleaned glass slides and let dried overnight at room temperature. While it was dried, fibrous PLGA films were prepared by thin polymer film dewetting under DI-water. By AFM scanning, the strips had thin (60 nm) and thick (~1 µm) region. To maintain fibrous structure on the glass slides after dewetting, two end sides against the pattern direction were secured by a nail polish. By immersing the imprinted films on glass slides in 50 ºC of DI-water for 1 h, the thin layers between thick regions were removed by dewetting. The fibrous sheets in water bath were cooled down to room temperature, and then lifted to the water interface (~25 °C) using forceps (Figure 3.3). The fibrous sheets were picked up by the agarose-coated substrates and let it dry 10 min and carefully cut the nail polish secured area off. The two sides along the direction of strips of the films were secured using 5 wt % PLGA solution in acetone after drying them at room temperature. The samples were placed at the 45 °C vacuum oven to remove residual solvent overnight.

Figure 3.3 Generation of fibrous PLGA sheets on agarose-coated surfaces.
3.3.4 Cell/PLGA Bundle Formation

The polymer sheets on agarose-coated substrates were placed in 24-well culture plates (USA Scientific) and sterilized for 15 min by exposure of UV light at a laminar hood (1300 series A2, Thermo Scientific). We used mouse embryonic fibroblast cells (MEFs, Millipore, Billerica, MA) as a model cell with a seeding density of $1 \times 10^5$ cells in 1 mL of a culture medium, which contained 85 v % DMEM cell medium and 15 v % fetal bovine serum (Invitrogen). After seeding cells, the cell culture plate was carefully shaken to regularly distribute the cells and placed in an incubator (NU-5500/E, NuAire) with 5 % CO$_2$. Morphologies and cell/polymer bundle formations of the sheets were observed under an optical microscope (IX81, Olympus).

To verify cell/polymer bundle formation for other types of cells ($1 \times 10^5$ cells in 1 mL), four different cell types were also used including rat induced vascular progenitor cells (iVPCs), human embryonic kidney 293A cell line (293A, ATCC, Manassas, VA), human liver cell line (HepG2, ATCC, Manassas, VA), and rat endothelial cells (ECs, Cell Applications, San Diego, CA). Cell behaviors on the films and the bundle formation during incubation were observed using an optical microscope.
3.4 Cell/Polymer Micro Bundles as a Noble Injection based Scaffold to Enhance Cell Viability and Retention

In vitro and in vivo evaluations of cell/polymer micro-constructs are described in this part. The detailed procedures are included, injectability, viability, proliferation, and retention of the micro-construct, and staining cells.

3.4.1 MTT Assay on Various Substrates

MEFs were seeded and incubated for 18 h on several surfaces as described above including plat-PLGA, PEG-silane modified glass slides, agarose-coated glass slides, and the pre-treated culture dish. The proliferation of MEFs on these surfaces was evaluated with a MTT cell proliferation assay (Invitrogen) following the protocol of the manufacturer. The incubated substrates were transferred to a new 24 well plate containing 500 µL of a culture medium (free of phenol red) and add 50 µL of MTT stock solution (12 mM MTT reagent in PBS). It was incubated for 4 h to allow the MTT reduction to MTT formazan at 37 °C incubator, and then to each well, 500 µL of SDS solution (2.5 g of SDS in 25 mL of 0.01 M HCl in PBS) was added. The dissolution of MTT formazan was allowed for 18 h incubation in the 37 °C incubator. The final solution was pipetted thoroughly and ~200 µL of the solution was moved into a 96 well plate. The absorbance of the final solutions was measured (wavelength: 570 nm) using SpectraMAs M4 Multi-mode microplate reader (Molecular Devices, Sunnyvale, CA).
3.4.2 Feasibility of Bundle Injection

The feasibility of cell/polymer bundle injection (incubated for 1 ~ 3 days) was verified using 1 mL of syringe with a G 20 needle (D: 584 µm) (Becton, Dickson and Company) and our design prepared by cutting ~1 cm of the end of 1mL pipette tip (US Scientific) with the same needle. The bundles were cut into 1 mm using surgical scissors and placed with 500 µL of the cell culture medium in a syringe. The needle was inserted and gently tapped to distribute bundles, and then the bundle/cell medium mixture was injected with a speed of ~2.2 mL/min in a petri dish. ~30 bundles were used for each test set and the percentage pass rate was obtained by counting the numbers inserted and ejected bundles from 30 tests. To evaluate the injectability with respect to the length of the bundle (~0.5, ~1, and ~2 mm) were obtained by cutting the bundles. The % pass rate was obtained as described above.

3.4.3 Preparation of Alginate Gel Environment

Alginate gel environment was prepared by carefully injecting 5 mL of 1 wt % alginate solution (cell culture medium (70 v %) and PBS (30 v %)) in 5 mL of 1 wt % CaCl₂ solution in PBS contained 6 well culture plate. Four G unit of alginate was cross-linked with Ca²⁺ by replacing Na⁺ on the alginate. After 1 min of reaction, unreacted solutions were discarded and the gels were rinsed 3 times with PBS. 5 mL of cell culture medium was added in the gel contained culture plate and let it for 1 h to exchange the fresh cell culture medium and the solution in alginate gel. In our preliminary studies, gelation within 1 min resulted in soft gels while hard gels were resulted if gelation exceeded more than 2 min. In the case of hard gels, significant leakage was observed,
while the leakage was marked decreased using the soft gels. Therefore, soft-alginate gel environment was utilized in this study.

3.4.4 Viability and Proliferation of Cell/Polymer Bundle Injection

Viabilities of cell polymer bundles and free cell injection were assessed in terms of before and after injection with a G 20 needle and 14 days after injection in an alginate gel environment as an in vitro model. The number of cells delivered in the alginate gels by bundle or free cell injection was $3 \times 10^5$.

3.4.4.1 Number of cells on bundles

To equivalent the number ($3 \times 10^5$) of cells on bundles, MEFs ($1 \times 10^5$) were incubated for 3 days on fibrous PLGA sheets (on agarose-coated surfaces). The bundles were cut into ~1 mm. The bundles with culture medium (5–8 mL) were corrected in a 50 mL centrifuge tube and 30 mL of PBS was added to dilute cell culture medium. It was centrifuged (1500 RPM for 5 min, X1R, Thermo Scientific) and the solution was discarded. 300 µL of 1X trypsin was added to the centrifuge tube to trypsinize for 5 min at 37 °C incubator, and then 4 mL of cell culture medium was added and gently pipetted to dilute the trypsin. The solution was discarded after centrifuging it again with the same procedure. After adding 4 mL of fresh cell culture medium, 20 µL of the cell suspension was mixed with 20 µL of 1X trypan blue, and dead and live cells were counted with aid of a hemocytometer under an optical microscope. 357 ± 22 bundles obtained from 8.5 samples were equivalent to $3 \times 10^5$ (estimated by using the trypan blue exclusion method
above). The number of cells on one bundle was estimated to $843 \pm 53$ cells by dividing the total number of cells by the number of the bundles. Total number of test sets was 5.

3.4.4.2 Proliferation and viability in alginate gels for 14 days

Bundles or dissociated cells were delivered using our design of injection with a G20 needle in alginate gels to assess the viability for bundle and free cell injection for 14 days. After injecting bundles or dissociated cells, the inserted area was secured by adding 100 $\mu$L of 1 wt % of CaCl$_2$ solution and rinsed with PBS 3 times. The gel contained culture plate was incubated after adding 3 mL of fresh cell culture medium and exchange the cell culture medium 3 days period. To detect the initial number of cells and viability in gels, each of the gel was moved in a 50 mL centrifuge tube contained a 10 mL of 10 wt % solution. After dissolving the gel for 10 min, it was centrifuged down 1500 RPM for 5 min) and the initial number of cells and viability of bundle and free cell injection was obtained by trypan blue exclusion (averaged from three tests). Viability and number of cells in alginate gels were obtained from 3 different gels at 0 (initial), 3, 7, and 14 incubation days.

3.4.5 Live/dead Cell Staining

To visualize live and dead cells, fluorescent staining (live: fluorescein diacetate (FDA) and dead: propidium iodide (PI), Sigma-Aldrich) was used as described manufacturer’s manual. Briefly, 6 $\mu$L of 5 mg/mL FDA solution in acetone and 4 $\mu$L of 1 mg/mL PI solution in water were added to 2 mL of cell culture medium containing cell/polymer bundles. After 3 min incubation the medium was discarded and PBS
solution was added to keep hydrated. Florescent images were obtained using a fluorescent microscope (Olympus IX81).

3.4.6 Retention Study using Alginate Gel in vitro Model

The Retention of cell/polymer bundle and free cell injection was studied using alginate gel as an in vitro environment. 5 wt % of thick alginate (Alginic acid sodium salt, Sigma) solution in PBS was prepared by heating at a microwave. The solution was sterilized in an autoclave for 15 min. To obtain 1 wt % of alginate gel in cell medium, the prepared 5 wt % mixture was diluted with cell medium by the volume ratio of 1:4 (mixture : cell medium). 5 mL of the 1 wt % alginate solution in cell medium was added to 5 mL of 1 wt % CaCl$_2$ (EMD millipore) solution in a 6 well plate for 1 min. After the gelation, the CaCl$_2$ solution was discarded using an aspirator and PBS was added to remove unreacted Ca$^+$ ions after rinsing several times. After 15 min, the PBS was discarded and 3 mL of cell medium was added. To test retention and viability of bundles for 7 days, bundles were injected in the alginate gel using a G 20 needle and the penetrated place was secured with small amount (~100 $\mu$L) of the CaCl$_2$ solution. The samples were placed in an incubator and images were taken regularly to observe its retention. Retention study of free cell injection was also studied similar to that of bundles injection. ~5 bundles as a position marker were injected at the middle of an alginate gel and $3 \times 10^5$ cells/500 $\mu$L was injected where bundles were injected or beneath of the bundles (bottom of the gel). After 3, 7, and 14 days, the gel was dissolved in 10 wt % of sodium citric acid, and evaluated its viability using trypan blue as it described in the section 3.4.4.
3.4.7 *In vivo* Assessment of Injected Cell/Polymer Micro Construct

The injectability of fibrous micro-constructs *in vivo* was preliminary demonstrated by injecting the constructs into a normal rat. In general, ~150 iVPC/polymer micro-constructs consisting of ~one million cells, or one million free iVPCs were used for each injection. The constructs or free iVPCs dispersed in 0.25 mL of culture medium were intramuscularly injected into the myocardium of a Sprague Dawley® (SD) rat (Charles River Laboratories International, Inc, Wilmington, MA) within 30 min after ligation by a G 20 needle following the animal protocol approved by IACUC from the Northeast Ohio Medical University (NEOMED). To localize the injected cells and micro-constructs, the cells were labeled with Td-tomato fluorescence by lentivirus (Life Technologies). 4 weeks after injection, the rats were sacrificed and heart tissues were harvested, sectioned and imaged using the fluorescent microscope (Olympus IX71).
3.5 Fully Biodegradable Injectable Tubes via Self-Rolling Bilayer Films for Cardiovascular Disease

In this part, fabrication of tubes via self-rolling bilayer films and characterization for injectable carriers are described. The detailed procedures include fabrication of bilayer films, cell encapsulation and viability, injectability, and pre-formed blood vessel generation.

3.5.1 Fabrication of Bilayer Films and Tubes Formation via Self-Rolling Bilayer Films

Flat PDMS stamps were prepared from Sylgard® 184 (Dow Corning, Midland, MI). Pre-polymer mixture was poured on a cleaned Si-wafer (Silicon Quest International, Reno, NV), de-gassed, and cured at 60 °C for 4 h. Fully cured PDMS sheet was cut to 2.5 cm \( \times \) 2 cm for the bottom stamp and 2.5 cm \( \times \) 1 cm for the upper stamp and placed on glass slides of the same size (Fisher Scientific, Waltham, MA). Figure 3.4 summarizes the fabrication of bilayer films. 20 µL of 3 ~ 10 wt.% of PLGA (Sigma-Aldrich, \( M_w = 35 \text{–} 60 \text{ kg/mol}, \text{Viscosity (i.v.)} = 0.45 \text{–} 0.60 \text{ dl/g}, \text{St Louis, MO} \) solutions in acetone (Fisher) was pressed (2 kgf/cm\(^2\)) in between PDMS sheets. The pressure was maintained for \(~2\) min, and the upper layer of PDMS sheet was carefully removed. The generated films were pressed on PDLA (PDLA, Polysciences Inc, \( M_w = 15 \text{ kg/mol}, \text{Viscosity (i.v.)} = 0.15 \text{–} 0.30 \text{ dl/g}, \text{Warrington, PA} \) films, which were spin-coated (200 RPM) using a spin coater (P-600, Specialty Coating Systems) with 3 ~ 9 wt.% of PDLA solutions in toluene (Fisher) on glass slides (1cm \( \times \) 1cm) and dried at 45 °C in the vacuum oven (model 1400E, VWR) overnight.
3.5.2 Fabrication of Several Types of Bilayer Films

The prepared bilayer films were cut into several aspect ratios (2:1, 4.5:1, and 8:1) of rectangular patterns with fixed width (500 µm). PLGA films with parallel strips were prepared by imprinting using featured PDMS (spacing ~20 µm), which was fabricated by fracture-induced structuring method we developed. Fabrication of bilayer films was the same as explained above. 8:1 aspect ratio of rectangular bilayer patterns was obtained by cutting with a razor blade. By cutting the bilayer films with different directions, parallel, diagonal, perpendicular patterns on the bilayer films to the long side of the bilayer films were prepared. Thicknesses of PLGA and PDLA films were determined by atomic force microscopy (AFM, Multimode NanoScope V, Veeco, Plainview, NY) and an ellipsometer (model 439, Rudolph Instrument), respectively. Tube width (averaged from 20 tubes) was measured from images taken by an optical microscope. The prepared films were exposed to UV light for 15 min in a laminar hood and the tube formation was achieved by incubating cell culture medium with and without cells for up to 7 days at a 5 % CO₂ incubator (NU-5500/E, NuAire).

Figure 3.4 Fabrication of bilayer films. PDLA films were spin-coated and PLGA films were generated in between PDMS pads. Bilayer films were obtained by pressing the PLGA films on PDLA coated substrates.
3.5.3 Fabrication of Strained PLGA Films by Bending

Three different bending strains on the PLGA films were applied using three different outer diameters (OD) of circular objects. PLGA films were imprinted as described above. The film attached to a release PDMS sheet was bent using several diameters of circular shapes: 20 mL vial (OD = 2.5 cm), 9 cm petri-dish (OD = 9 cm), and 14 cm petri-dish (OD = 14.2 cm), which resulted in ~5, ~3, and ~2 % strains ($\varepsilon_x$), respectively. To measure the bending strains, two points towards the bending direction were marked on the PDMS sheet and lengths of the initial and after bending were measured. The resulted % bending strains were obtained from the length of initial and after bending. Bent film transferred to PDLA films (thickness: 330 nm) on glass slide by rolling from one side to another with maintaining the pressure (2 kgf/cm$^2$). The bilayer films were dried at 45 °C in the vacuum oven (model 1400E, VWR) overnight. An aspect ratio of 8:1 rectangular patterns (L: 4 mm, w: 500 µm) was achieved by cutting with a razor blade. The cutting direction of length of the bilayer films was perpendicular to the bending direction.

3.5.4 Fabrication of patterned bilayer films

Parallel patterns on bilayer films were fabricated by imprinting of 20 µL PLGA solution (9 wt %) in acetone in between a flat PDMS sheet and a featured PDMS stamp (the detailed fabrication of the featured PDMS stamp is described in sec. 3.3.3). After releasing the PDMS stamps (PLGA films were attached on the featured stamp due to the large contact area), the featured PDMS stamp was pressed to PDLA film (330 nm) on glass slide. The patterned bilayer films were dried at 45 °C in the vacuum oven (model
1400E, VWR) overnight. And 8:1 aspect ratio of rectangular bilayer patterns was obtained by cutting with a razor blade. By cutting the bilayer films with different directions, parallel, diagonal, perpendicular patterns on the bilayer films to the long side of the bilayer films were prepared.

3.5.5 Cell Encapsulation and Viability Studies

Mouse embryonic fibroblasts (MEFs, Millipore, Billerica, MA), mouse endothelial cells (ECs, Cell Applications, San Diego, CA), mouse smooth muscle cells (SMCs, Cell Applications, San Diego, CA) were seeded (1 × 10^5 cells in 1 mL of culture medium) on bilayer films. To measure the viability during incubation for 7 days, trypan blue (Sigma-Aldrich) was initially utilized. However, in the case of SMCs and ECs, total number of cells was unable to be counted since they were well spread inside the tubes. Therefore, CellTracker™ Green CMFDA (Life technologies) and Hoechst Blue (Life Technologies) were stained before seeding cells. The number of live cells was counted and the total number of cells was obtained by counting nuclei (stained with Hoechst Blue) to verify viability. To stain cells, the cells were grown until it reached confluent level. Cell culture medium was removed and rinsed (2 times) with 4 mL of PBS (Invitrogen). 4 mL of 20 μg/mL of CellTracker™ Green CMFDA and 5 μg/mL of Hoechst Blue in the serum free media (DMEM, Sigma-Aldrich) was mixed together and poured in the plates and incubated for 45 min. The dyeing solutions were discarded and rinsed (2 times) with 4 mL of PBS. 4 mL of fresh cell culture media was added to the culture dish and it was incubated for 30 min. According to manufacture’s description, more than 3-day track is
possible. In our case, cells incubated for 7 days showed decreased intensity compared to the intensity after 3 days incubation, but still it was possible to track their viability.

Total number of cells was counted from florescent images of nuclei stained with Hoechst Blue and viable cells were counted from florescent images of cells stained with CellTracker™ Green CMFDA. Bright field and florescent images were taken using optical microscope (Olympus IX71, B&B Microscope, Pittsburg, PA). As a negative control, dissociated cells were seeded with the same density on an agarose coated 24-well plate (USA Scientific Inc, Ocala, FL). To generate agarose (UltraPure™ agarose, Life Technologies, Carlsbad, CA) coated surfaces, 1 mL of 1 wt % agarose solution in PBS was added on a 24 well plate and dried over night at room temperature. The viability was obtained by counting live and dead cells after staining it with trypan blue from ~ 20 samples.

3.5.6 Feasibility of Injection

Injectability was verified using three different lengths of tubes (~1, ~2, and ~4 mm) incubated for 3 days. Two different types of injection were utilized; one was conventional injection (BD, Franklin Lakes, NJ) and our in-house design with the G 20 needle (BD) (Figure 3.5). We developed injection to induce laminar flow through the needle by cutting the end of a pipette tip (1 mL, USA Scientific). About 30 bundles were picked up with culture medium and injected with 2.2 mL/min. % pass ratio was verified by counting of ejected tubes.
Figure 3.5 Two types of injection for injectability study: (a) Conventional injection and (b) in-house design of injection.
3.5.7 Cell Alignment Characterization

Flat and patterned surfaces were prepared as described above (sec 3.3.2 and sec. 3.3.3). SMCs ($1 \times 10^5$ cells/mL) were seeded on the surfaces and incubated for 20 h. Eight separate regions of each sample were imaged and the images were analyzed using ImageJ NIH image processing software (Bethesda, MD). An ellipse was drawn with respect to the long and short axis of an elongated cell. Alignment of cells was considered if the angle between the long axis and the pattern direction less than 15 ° (Figure 3.6). For each experiment, the percent of cell alignment was determined by counting ~100 cells.

Figure 3.6 Characterization of cell alignment on patterned surfaces.
3.5.8 Co-culture of SMCs and ECs on Bilayer Films

Prior to co-culture of SMCs and ECs, their encapsulation and viability for 7 days in tubes were verified using flat bilayer films with aspect ratio of 8:1. To develop the pre-formed blood vessels, diagonal patterned bilayer films (2:1, 4:1, and 8:1) were utilized. Prior to seeding the cells, SMCs and ECs were stained with CellTracker™ Green CMFDA and Hoechst Blue, respectively to verify encapsulation of two different cells in tubes as described in sec. 3.5.2. 1 mL of $5 \times 10^4$ SMCs were seeded on bilayer films and the same number of ECs were seeded before the rolling started. The bilayer films started rolling after 4 h, we decided to seed ECs at that point. Bright field and fluorescent images were taken by a microscope and confocal images were obtained using a laser scanning confocal microscope (Olympus FluoView™ FV1000).

3.6 Statistics / Data Analysis

All results are an average of at least three replicates and presented as mean ± standard deviation (SD). Statistical analysis was performed to evaluate the statistical significance by analysis of variance (ANOVA, XLSTAT software) with post hoc Turkey testing for multiple comparisons. Statistical significance for $p < 0.05$ and $p < 0.001$ were denoted by * and **, respectively.
CHAPTER IV
RESULTS AND DISCUSSION

This chapter summaries all the results and discussion of this work. It consists of four sections, which correspond to the fabrication of two different types of scaffolds and its characterization. Section 4.1. describes dewetting behaviors of poly(lactic-co-glycolic acid) thin films under aqueous media, which are fundamental phenomenon to be utilized for further fabrication of the scaffolds. Section 4.2. demonstrates the fibrous polymer bundle formation via thin polymer film dewetting. Section 4.3. discusses the cell/polymer micro-bundles as a noble injection scaffold to enhance cell viability and retention. Section 4.4. details the fully biodegradable injectable tubes via self-rolling of bilayer films for possible further use in the treatment of cardiovascular disease.

4.1 Dewetting of Poly(D,L-lactic-co-glycolic acid) Thin Films under Aqueous Media

This part demonstrates the results and discussions about dewetting behaviors of PLGA thin films under aqueous solutions. The subsections include introduction, surface tension/energy and their components, dewetting of PLGA thin films under aqueous solutions, potential source of instabilities, and dewetting suppression under protein containing solutions, and summary.
4.1.1 Introduction

Polymer films have been increasingly utilized for a variety of biomedical applications. The examples include biocompatible coatings for implants,\textsuperscript{126,127} scaffolds for tissue engineering,\textsuperscript{128,129} carriers for drug delivery\textsuperscript{130,131} and gene therapy\textsuperscript{132,133}. For most of these applications, thin films are submerged in or in contact with a liquid medium (e.g. culture medium, PBS), and dewetting of these thin films, could greatly affect their utilization. However, dewetting of biocompatible or biodegradable polymer thin films under physiological conditions has rarely been investigated.

Dewetting of a polymer thin film (< 100 nm) occurs when the film is heated above the glass transition temperature ($T_g$) of the polymer, and sufficient van der Waals attractions across the film making the film unstable. Thin film dewetting in air is most common and widely studied;\textsuperscript{115,116,134–139} polymer thin film dewetting under a liquid has also been reported by Verma and Sharma.\textsuperscript{139,140} They studied thin polystyrene film dewetting under a solvent/water mixture and utilized such dewetting for fabricating submicron-sized lenses with high contact angles. They suggested that dewetting of these thin films under liquids could mainly be controlled by electrostatic interactions, which is not normally observed for dewetting under ambient conditions. According to Reiter,\textsuperscript{118,119} dewetting initiated with the formation of dewetted holes in the film, and the number of holes ($N_{hi}$) vs. thickness of the films ($h$) could be plotted into a power law relationship, and the exponent of the fit elucidated the governing factor behind the instability (i.e. dewetting) of the film.
In this study, we evaluated dewetting of poly(lactic-co-glycolic acid) (PLGA) thin films under two common aqueous solutions, PBS and cell medium, used in cell culture applications, and with DI-water and BSA/PBS solution served as their respective control. PLGA is a widely utilized biodegradable polymer, and scaffolds made out of PLGA thin films or particles are most frequent models for both fundamental and applied studies in biomedical field. The effect of supporting substrate on dewetting of PLGA films under liquid medium was also assessed. For each condition, $N_{th}$ vs. $h$ was plotted to roughly elucidate the governing factors behind the initiation of the instabilities in aqueous solutions, which could potentially provide us a mean to control the dewetting of PLGA thin films under liquid media.

4.1.2 Surface Tension/Energy and Their Components

The surface energy of substrates and surface tension of liquids were determined using measured contact angle values (Table 4.1) and liquid–liquid interfacial tension values (Table 4.2), respectively. These values are summarized in Table 4.3, along with the surface energy of bovine serum albumin (BSA) reported by others.\textsuperscript{141}

The surface energies of PLGA, Si-wafer and the PEG-silane modified Si-wafer were estimated to be 47.2 mJ/m$^2$, 46.4 mJ/m$^2$ and 45.4 mJ/m$^2$, respectively. For these three surface, their surface energy was mainly contributed by their $\gamma_{LV}$. For PLGA, Si-wafer and the PEG-silane modified Si-wafer, the values of $\gamma_{LV}$ were 43.8 mJ/m$^2$, 43.9 mJ/m$^2$ and 45.4 mJ/m$^2$, respectively. The values of $\gamma_{AB}$ were 3.5 mJ/m$^2$ for PLGA, 2.5 mJ/m$^2$ for Si-wafer, and 0 mJ/m$^2$ for the PEG modified Si-wafer. All three surfaces had
much higher $\gamma^-$ than $\gamma^+$, which could be due to the presence of --COOH groups or --OH groups on the surface.

Table 4.1 The measured contact angles of DI-water, methylene iodide and ethylene glycol form on the PLGA film, Si-wafer and the PEG modified Si-wafer.

<table>
<thead>
<tr>
<th>Contact angle (°)</th>
<th>DI-water</th>
<th>methylene iodide</th>
<th>ethylene glycol</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA</td>
<td>61.7 ± 0.6</td>
<td>34.2 ± 0.8</td>
<td>39.5 ± 0.4</td>
</tr>
<tr>
<td>Si-wafer</td>
<td>15.9 ± 1.1</td>
<td>35.0 ± 0.4</td>
<td>13.6 ± 0.9</td>
</tr>
<tr>
<td>PEG modified Si-wafer</td>
<td>34.0 ± 0.6</td>
<td>19.8 ± 0.4</td>
<td>20.9 ± 0.4</td>
</tr>
</tbody>
</table>

Table 4.2 The measured interfacial tensions between the four aqueous solutions and three organic solvents: ethyl acetate, ethyl ether and benzaldehyde.

<table>
<thead>
<tr>
<th>Interfacial tension (mJ/m²)</th>
<th>ethyl acetate</th>
<th>ethyl ether</th>
<th>Benzaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI-water</td>
<td>6.8 ± 0.2</td>
<td>10.7 ± 0.2</td>
<td>16.0 ± 0.3</td>
</tr>
<tr>
<td>PBS</td>
<td>6.4 ± 0.2</td>
<td>11.3 ± 0.2</td>
<td>13.9 ± 0.2</td>
</tr>
<tr>
<td>0.4 % BSA in PBS</td>
<td>5.9 ± 0.3</td>
<td>10.5 ± 0.3</td>
<td>7.7 ± 0.5</td>
</tr>
<tr>
<td>Cell culture medium</td>
<td>6.1 ± 0.3</td>
<td>10.3 ± 0.3</td>
<td>8.6 ± 0.4</td>
</tr>
</tbody>
</table>
The surface tensions determined for the four liquids were 73.3 mJ/m$^2$ for water, 79.6 mJ/m$^2$ for PBS, 67.1 mJ/m$^2$ for BSA in PBS, and 64.7 mJ/m$^2$ for the cell culture medium, and their $\gamma^{\text{LW}}$ values were, respectively, 21.9 mJ/m$^2$, 28.0 mJ/m$^2$, 38.7 mJ/m$^2$ and 35.6 mJ/m$^2$. For $\gamma^{\text{AB}}$, water and PBS had similar values, i.e. 51.4 mJ/m$^2$ and 51.6 mJ/m$^2$, respectively; the BSA/PBS and cell culture medium also had similar, but much lower values of $\sim$ 29 mJ/m$^2$, which was the result of the lower value of $\gamma^{+}$ ($\sim$ 7.6 mJ/m$^2$) as compared to those of water and PBS ($\sim$ 25.0 mJ/m$^2$).

Table 4.3 The surface energy and its components estimated based on the contact angle and interfacial tension values for the various materials used in this study.

<table>
<thead>
<tr>
<th>Materials/Liquids (mJ/m$^2$)</th>
<th>$\gamma$</th>
<th>$\gamma^{\text{LW}}$</th>
<th>$\gamma^{\text{AB}}$</th>
<th>$\gamma^{+}$</th>
<th>$\gamma^{-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA</td>
<td>44.1</td>
<td>42.4</td>
<td>1.7</td>
<td>0.039</td>
<td>19.4</td>
</tr>
<tr>
<td>Si-wafer</td>
<td>45.3</td>
<td>42.0</td>
<td>3.3</td>
<td>0.042</td>
<td>63.1</td>
</tr>
<tr>
<td>PEG modified wafer</td>
<td>47.6</td>
<td>47.5</td>
<td>0.1</td>
<td>0.000</td>
<td>46.3</td>
</tr>
<tr>
<td>BSA$^a$</td>
<td>46.2</td>
<td>39.5</td>
<td>6.7</td>
<td>3.7</td>
<td>3.0</td>
</tr>
<tr>
<td>DI-water</td>
<td>73.3</td>
<td>21.9</td>
<td>51.4</td>
<td>26.0</td>
<td>25.4</td>
</tr>
<tr>
<td>PBS</td>
<td>79.6</td>
<td>28.0</td>
<td>51.6</td>
<td>26.7</td>
<td>24.9</td>
</tr>
<tr>
<td>0.4% BSA in PBS</td>
<td>67.1</td>
<td>38.7</td>
<td>28.5</td>
<td>7.7</td>
<td>26.3</td>
</tr>
<tr>
<td>Cell culture medium</td>
<td>64.7</td>
<td>35.6</td>
<td>29.1</td>
<td>7.6</td>
<td>27.9</td>
</tr>
</tbody>
</table>

Note: $^a$: Values were obtained from Ref. [141].
4.1.3 Dewetting of PLGA Thin Films under Aqueous Solutions

Dewetting of PLGA films under DI-water, PBS, BSA/PBS, and cell culture medium, was followed. Since in many applications, a non-fouling surface, such as a PEG surface, is more desired to serve as a support to place the polymer films on, dewetting of PLGA films on the PEG-silane modified Si-wafers under BSA/PBS and culture medium was also evaluated. Figures 4.1 and 4.2 summarize the progressive dewetting stages of PLGA films with a thickness of ~30 nm (Figure 4.1) and of ~70 nm (Figure 4.2). The general stages, from nucleation of holes to breaking down of the dewetted films into droplets, involved in dewetting of thin PLGA films under aqueous solutions were basically the same, which also followed the typical dewetting phenomenon observed in air.\textsuperscript{115,116} When observed closely, some difference in the details of PLGA thin film dewetting under different aqueous solutions was noticed. Dewetting of PLGA films under PBS initiated and proceeded the fastest, closely followed by that under DI-water, and the slowest under BSA/PBS and culture medium (Figure 4.3). The cleanest hole morphology (e.g. with the smoothest hole rims/edges) was observed for PLGA film dewetted under PBS. Under BSA/PBS and cell culture medium, the films on the PEG modified Si-wafer showed a much slower dewetting than that on a non-treated Si-water. Since dewetting of PLGA films under BSA/PBS and cell culture medium was found to be very similar, the results in culture medium were then presented for more detailed comparisons and analyses.

For relatively thin films (~30 nm), the morphological changes of thin films under different conditions at 2, 6, 24, and 96 h were shown in Figure 4.1. Under PBS, at ~2 h,
the maximum number of holes (i.e. hole density), immediate prior to their coalescent with each other, in films supported on Si-wafer was found. Also, the holes had no detectable rims. After 6 h of incubation, most holes had merged with each other to form polygon structures, and the films were found to be slightly detached from the substrate. The polygon morphology remained throughout the rest of incubation period (up to 96 h). Under DI-water and cell medium, with Si-wafer as the substrate support, it took ~6 h of incubation for the hole density to reach maximum. After 24 h, dewetting of PLGA film from Si-wafer under both DI-water and cell culture medium proceeded to form polygons. After 96 h, films under DI-water had completely broken down into droplets, whereas suppression of dewetting was noticed in the cell culture medium condition. For films supported on the PEG modified Si-wafer and dewetted under BSA/PBS solution and culture medium, similar results were observed, the number of holes never appeared to reach maximum (Figures 4.1 and 4.3), and the hole size only increased slightly (from ~10 \( \mu \text{m} \) to ~17 \( \mu \text{m} \)) during the first 24 h, and then maintained through the rest of the experiment.
Figure 4.1 Time evolution of ~30 nm of PLGA films dewetting under various aqueous solutions. Films on Si-wafer in (a) water, (b) PBS, (c) cell culture medium, and (d) films on the PEG-modified Si-wafer in cell culture medium. Scale bar: 25 µm.
For relatively thick films (~70 nm), morphological images taken at 24, 96, and 192 h of incubation were summarized in Figure 4.2. Holes were observed after 24 h of incubation for all cases, and coalescent of holes under PBS had already occurred. Under PBS, a similar observation as that of thinner films was noticed, films were found to be slightly detached and maintained the polygon structures formed at 96 h of incubation. The detachment of PLGA films from the substrate could be caused by the faster penetration of liquid to the PLGA/substrate interface that weakened the interfacial adhesion. Since dewetting of PLGA films under PBS proceeded the fastest, as dewetting occurred, more substrate was exposed to allow easier penetration of PBS to the interface. Once detached, dewetting of PLGA films under PBS did not show further evolution (e.g., to form droplets).

Under DI-water, at ~96 h, dewetted holes had merged to form polygon shapes, but the features were somewhat different from those of the polygons observed under PBS, and further progressed polygons were observed at 192 h. Under BSA/PBS solution and cell culture medium, the dewetting holes of films supported on Si-wafer coalesced to form larger holes; while dewetting holes in the film supported on the PEG modified Si-wafer appeared much smaller and continued to initiate from 24 h to 96 h, and reached the maximum number at ~96 h. Additional hole initiation and coalesce of earlier initiated holes were observed for films on Si-wafer to form smaller and very irregular polygons. For films supported on the PEG-modified Si-wafer, the initiated holes only grew slightly (from ~15 µm to ~23 µm) from 96 h to 192 h, and they never coalesced to form polygons (Figures 4.2 and 4.3).
Figure 4.2 Time evolution of ~70 nm of PLGA films dewetting under various aqueous solutions. Films on Si-wafer in (a) water, (b) PBS, (c) cell culture medium, and (d) films on PEG modified Si–wafer in cell culture medium. Scale bar: 25 µm.
Figure 4.3 Time evolution of (a) ~30 nm and (b) ~70 nm of PLGA films dewetting under 0.4 w/v % BSA in PBS. Scale bar: 25 µm.
4.1.4 Potential Source of Instabilities

To reveal the potential source of instabilities of PLGA films under the aqueous solutions investigated, 10 to 100 nm thick PLGA films were dewetted. Films thinner than 12 nm were found to be fully dewetted within 15 min, and it was difficult to follow the dewetting process and determined the maximum hole density for these thin films, thus only holes formed in films thicker than 12 nm were considered. In addition, only ~3 different thicknesses of PLGA films were prepared in the range of 10 – 30 nm because fast evaporation of ethyl acetate (73 mmHg at 20 °C) during spin-coating made difficult to precise control of the thicknesses. Figure 4.4 shows the plots of the number of holes ($N_H$) vs. thickness ($h$) of PLGA films dewetted under different conditions. The slopes of the best fits of the double-logarithmic plots over the entire film thickness range (12 to 100 nm) were found to be $-3.9 \pm 0.3$ for the Si-wafer/DI-water combination, $-4.6 \pm 0.3$ for the Si-wafer/PBS combination, $-4.4 \pm 0.6$ for the Si-wafer/cell culture medium combination, and $-5.1 \pm 0.8$ for the PEG modified Si-wafer/cell culture medium combination. However, the data points were scattered. In an attempt to obtain more meaningful information from the data, the thickness ranges were divided into a thinner (< 20 nm for cell culture medium and < 30 nm for DI-water and PBS) region and a thicker (> 20 nm for cell culture medium and > 30 nm for DI-water and PBS) region. The division was made based on the most dramatic value change for each case. Figure 4.4 shows the slopes of the double-logarithmic plots obtained on the two regions. In the thinner region, the powers (i.e. slopes) were $-3.3 \pm 0.2$, $-3.4 \pm 0.2$, and $-3.2 \pm 0.3$, respectively, for the films dewetted from Si-wafer under DI-water, PBS, and culture medium; while they were $-3.8 \pm 0.2$, $-3.9 \pm 9.2$, and $-2.7 \pm 0.7$ for the thicker region.
For PLGA thin films dewetted from the PEG-silane modified Si-wafer under culture medium, the powers were $-2.9 \pm 0.1$ and $-3.2 \pm 0.4$, respectively, for the thinner and the thicker regions; both values were close to $-3.0$.

Figure 4.4 Double-logarithmic plots of the maximum number of holes ($N_{HI}$) as a function of PLGA film thickness ($h$) in aqueous solutions. For dewetting under (a) DI-water and PBS, the slopes of the best fit lines are $-3.9 \pm 0.3$ ($h$: 10–100 nm), $-3.3 \pm 0.2$ ($h < 30$ nm), and $-3.8 \pm 0.2$ ($h > 30$ nm) for DI-water and $-4.5 \pm 0.3$ ($h$: 10–100 nm), $-3.4 \pm 0.2$ ($h < 30$ nm), and $-3.9 \pm 0.2$ ($h > 30$ nm) for PBS. (b) For dewetting under cell culture medium, the slopes of the best fit lines are $-4.4 \pm 0.6$ ($h$: 10–100 nm), $-3.2 \pm 0.3$ ($h < 20$ nm), and $-2.7 \pm 0.7$ ($h > 20$ nm) for PLGA on Si-wafer and $-5.1 \pm 0.8$ ($h$: 10–100 nm), $-2.9 \pm 0.1$ ($h < 20$ nm), and $-3.2 \pm 0.4$ ($h > 20$ nm) for PLGA on PEG-modified wafer.
According to earlier studies, the maximum number of holes ($N_H$) is inversely proportional to the square of the wavelength ($\lambda$) (i.e. $N_H \sim \lambda^{-2}$) of the long-wave instability of spinodal dewetting, which is defined as:

$$\lambda = [-8\pi^2 \gamma / (\phi'(h))]^{1/2}$$  \hspace{1cm} (15)

where, $\phi (h)$ is the effective interface potential and $\gamma$ is the interfacial tension. According to equation (15), $\gamma$, $\phi$, and $h$ are main parameters affecting film instability. Interactions between surfaces normally consist of van der Waals interactions, acid-base interactions, and electrostatic interactions. Acid-base interactions (e.g. hydrogen bonding) are short-ranged ($< 10$ nm), thus can be neglected in our dewetting study. $\phi$ would then vary from van der Waals attractions to electrostatic interactions depending on the circumstance. In the case of van der Waals attractions, $\phi = A_e / 6\pi h^3$, equation (15) becomes:

$$\lambda_{vW} = 4\pi \frac{\pi \gamma h^2}{\sqrt{A_e}}$$ \hspace{1cm} (16a)

where $A_e$ is the effective Hamaker’s constant. For electrostatic interactions, $\phi = \varepsilon\varepsilon_0 U^2 / 2h^2$ and equation (4) becomes:

$$\lambda_{EL} = \frac{2\pi}{U} \sqrt{\frac{2\gamma h^{3/2}}{\varepsilon\varepsilon_0}}$$ \hspace{1cm} (16b)

As a result, if thin film dewetting is governed by van der Waals attractions, $N_H \sim \lambda^{-2} \sim h^{-4}$; whereas if film dewetting is governed by electrostatic interactions, $N_H \sim \lambda^{-2} \sim h^{-3}$.

If holes are appeared with regular separation distance, it could be simple to know a dominant length scale by directly measuring the distances between holes. It would not
be simple to find a dominant length scale since holes appear randomly (Figure 4.2 and 4.3). Therefore, we did not measure the separation distance. Instead, we utilized equation (15) and (16) with the relation of $N_H \sim \lambda^{-2}$. Based on our experimentally fitted $N_H$ vs. $h^n$, most of the $n$ values were around $-3$, indicating their dewetting was primarily dominated by electrostatic interactions, especially for thinner films ($< 30$ nm or 20 nm). Similar results were reported by Verma and Sharma,$^{139,140}$ where electrostatic interactions were reported to dominate the dewetting of polystyrene films under the mixture of water-acetone-methyl ethyl ketone. According to the authors, H$^+/\text{OH}^-$ ions unequally distributed on a polymer–liquid interface could be the main source of the surface charge and enhanced electrostatic interactions when polymer thin films were under a liquid. For thicker films, dewetting of PLGA films from Si-wafer under DI-water and PBS had an $n$ value close to $-4$, suggesting for those two cases, dewetting could be controlled by van der Waals attractions.

When long range van der Waals interactions across the film are attractive, i.e. a positive value of the Hamaker’s constant ($A$), the film is un-stable and the instability will finally lead to dewetting of the thin film. When $A$ is negative, the film is expected to be stable if only van der Waals interactions control thin film dewetting. The Hamaker’s constant for our systems (medium/polymer film/substrate) can be expressed as:

$$A = A_{\text{MPS}} = 24\pi l_0^2 (\sqrt{\gamma_M^{1W}} - \sqrt{\gamma_P^{1W}})(\sqrt{\gamma_S^{1W}} - \sqrt{\gamma_P^{1W}})$$  \hspace{1cm} (17)$$

Using apolar components in Table 4.3, $A_{\text{MPS}}$ were estimated (values reported in Table 4.4). For PLGA films on Si-wafer under DI-water and PBS, the estimated values
of $A_{\text{MPS}}$ were $1.05 \times 10^{-22}$ and $6.93 \times 10^{-23}$ J, respectively, indicating that the PLGA films were unstable under these conditions if dewetting was controlled by van der Waals interactions. Also, the values of $A_{\text{MPS}}$ corresponded to the spacing (e.g., wavelength) between the dewetted holes (see equation 16a) – a larger spacing (i.e., less number of holes) should be resulted with a smaller value of $A_{\text{MPS}}$, which was noticed experimentally under PBS.

In term of electrostatic interactions, zeta potentials of PLGA particles (average diameter: $700 \pm 200$ nm) under water and PBS were measured to be $-55.4$ mV and $-30.7$ mV (Table 4.5), respectively. If we set the zeta potential of the PLGA/Si-wafer interface as $\zeta_{\text{PS}}$, the exact value could not be experimentally measured, the potential difference in the case of water and PBS became, respectively, $(-55.4 - \zeta_{\text{PS}})$ mV and $(-30.7 - \zeta_{\text{PS}})$ mV. Therefore, electrostatic interactions across the PLGA film under water were stronger than those under PBS, indicating a smaller spacing between holes (following equation 16b) when films dewetted under water. This was also confirmed experimentally, where smaller spacing (i.e. more holes) was observed for PLGA films dewetted under DI-water.
Table 4.4 Hamaker’s constants ($A_{MPS}$) of a PLGA film on Si-wafer and the PEG modified Si-wafer in aqueous solutions.

<table>
<thead>
<tr>
<th>Liquid</th>
<th>Substrate</th>
<th>$A_{MPS}$ (J)</th>
<th>Protein adsorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI-water</td>
<td>Si-wafer</td>
<td>$1.05 \times 10^{-22}$</td>
<td>–</td>
</tr>
<tr>
<td>PBS</td>
<td>Si-wafer</td>
<td>$6.93 \times 10^{-23}$</td>
<td>–</td>
</tr>
<tr>
<td>0.4 % BSA in PBS</td>
<td>Si-wafer</td>
<td>$1.66 \times 10^{-23}$</td>
<td>$-2.33 \times 10^{-23}$</td>
</tr>
<tr>
<td>Cell culture medium</td>
<td>PEG modified</td>
<td>$-2.05 \times 10^{-22}$</td>
<td>$-7.22 \times 10^{-23}$</td>
</tr>
<tr>
<td></td>
<td>Si-wafer</td>
<td>$3.12 \times 10^{-23}$</td>
<td>$-1.16 \times 10^{-22}$</td>
</tr>
<tr>
<td></td>
<td>PEG modified</td>
<td>$-3.85 \times 10^{-22}$</td>
<td>$-3.59 \times 10^{-22}$</td>
</tr>
</tbody>
</table>

Table 4.5 Zeta potentials ($\zeta$) of PLGA particles in aqueous solutions.

| $\zeta$(mV) of PLGA particles in | $\zeta$(mV) of protein adsorbed PLGA particles in |
| DI-water          | PBS              | PBS              |
| $-55.4 \pm 2.4$  | $-30.7 \pm 2.1$  | $-18.9 \pm 1.3^a$ |
|                  |                  | $-14.9 \pm 1.8^b$ |

Note: $^a$: from 0.4 w/v % BSA in PBS; $^b$: from cell culture medium
4.1.5 Dewetting Suppression under Protein Containing Solutions

As described earlier, dewetting suppression of PLGA film under BSA/PBS solution and cell culture medium was noticed. This phenomenon was most likely due to the adsorption of proteins on the PLGA films. The adsorbed proteins could alter surface energy of polymer films. Therefore, we speculated that proteins adsorbed on the PLGA films changed the surface properties (e.g. surface energy and/or surface charge) of PLGA films, which resulted in the suppression of dewetting. The dewetting data (i.e. $N_H$ ~ $h^{-3}$), for the separated thin and thick regions, indicated that the governing factor behind the dewetting of PLGA films under culture medium was most likely electrostatic interactions. Since protein adsorption could occur within minutes, but the time to reach the maximum hole number would be in hours (> 6 h), the properties of protein adsorbed PLGA films would be more adequate to be utilized to interpret the dewetting behaviors of PLGA films under protein containing solutions.

The values of $A_{MPS}$ of proteins adsorbed PLGA films were both negative ($-2.33 \times 10^{-23}$ J under BSA/PBS and $-1.16 \times 10^{-22}$ J under culture medium), indicating that the film would be stable, i.e. the quickly adsorbed proteins suppressed dewetting of PLGA films under these protein containing solutions. Suppression of dewetting by changing the value of Hamaker’s constant from positive to negative has been reported by others. For example, CeO₂ nanoparticles segregated to the interface of air–polystyrene (PS) thin films (~50 nm) completely suppressed dewetting of the PS films due to the resulting negative Hamaker’s constant; while pure PS thin films (~50 nm) under the same conditions dewetted because of the positive Hamaker’s constant.
Altering electrostatic interactions could also change the destabilizing pressure of PLGA films. In our cases, zeta potentials of the protein adsorbed PLGA films were lower (−18.9 mV or −14.9 mV) than those of films without protein adsorbed (−30.7 mV), which would suggest smaller electrostatic interactions. The van der Waals repulsion, i.e. to keep the film stable, could be weaker than the weakened electrostatic interactions, and dewetting was able to slowly proceed (Figure c1 to c3) by electrostatic interactions, as seen experimentally.

Hamaker’s constants of protein adsorbed PLGA films on the PEG-silane modified Si-wafer, as compared to un-modified Si-wafer, were more negative in both BSA/PBS and culture medium, suggesting PLGA films would not dewet from the PEG-silane modified Si-wafer under protein containing solutions if dewetting was solely governed by van der Waals interactions. Thus, electrostatic interactions again governed the dewetting of PLGA films under BSA/PBS and culture medium, as experimentally observed.

4.1.6 Summary

Dewetting of thin PLGA films under DI-water, PBS, BSA/PBS and cell medium (films on Si-wafer and the PEG modified Si-wafer) were investigated. Dewetting of films was the fastest under PBS, but the slowest under BSA/PBS and cell medium, especially on the PEG modified support. Under DI-water and PBS, electrostatic interactions were found to be responsible for dewetting of PLGA films when they were thinner than 30 nm, but for thicker films (> 30 nm), van der Waals attractions dominated the film instability. Proteins adsorbed on PLGA films greatly changed surface properties
of these films, and reduced the destabilizing intermolecular potential (ϕ) for PLGA films under both BSA/PBS and culture medium. In these protein-containing solutions, dewetting of PLGA films was mainly governed by the weakened electrostatic interactions, and dewetting was greatly suppressed.
4.2 Fibrous Polymer Bundle Formation via Thin Polymer Film Dewetting

This part demonstrates the results and discussions about fibrous polymer bundle formation via thin polymer film dewetting. The subsections include introduction, proliferation on different surfaces, different stabilities of imprinted films under cell culture condition, the dewetting process of imprinting polymer thin films, cell/polymer bundle formation, and summary.

4.2.1 Introduction

Although injection of dissociated cells is considered to be a minimally invasive and a cost-effective method to deliver cells to defective host organs, low efficiency has been continuously reported.\textsuperscript{8-12} The delivered cells are expected to be retained at the target site and proliferate to fill the defects to restore the function of the tissue. However, they do not have extracellular matrix and the small size of cells resulted in easy relocation right after injection. It causes significantly low engraftment ratio with host organ within 1 week.\textsuperscript{12} For these reasons, polymeric scaffolds are widely utilized for fabricating injectable cell carriers to overcome such shortcomings of free cell injection. To be injectable, there are solution types (e.g. physical gels) or small sizes of particles, which encapsulate cells or are used to be supportive structures that cells can adhere. After injection into the host organ, the scaffolds play a role for retaining cells at the target site, offer supports for cell growth, and degrade in physiological conditions.\textsuperscript{13}

It has been reported that cell fates and functions are largely affected by the properties of polymeric surfaces.\textsuperscript{11} For example, topographic and chemical patterning
can induce the directional growth of cells, which is directly related to the function of tissues. To achieve similar tissue structures and functions from the delivered cells within the host organ, mimicking native structures and incorporating it to the polymeric scaffolds would be ideal. However, incorporating the structures found on the native tissue is hardly achieved by current approaches. For example, physical hydrogel are in solution state before injection and it could not generate tracks to guide cells. Fabricating injectable particles have been well established since it originate from drug delivery carriers, however, their shapes are mostly circular shapes, which could not be found on any of the native structures. Native tissues are mainly composed of fibrous structures, for example, neurons, muscles, blood vessels, etc. There are little studies to incorporate fibrous structures generated by electrospinning technique. Hsieh et al. showed potential injectable carriers, which was electrospun poly(ε-caprolactone-co-DL-lactide) mixed with physical hydrogel precursor. The electrospunned fibers were sonicated to break into smaller pieces to be injectable and blended with hydrogels. According to their results, neuron stem cells differentiated into neurons. Similar approach was reported by Coburn et al. poly(ε-caprolactone) (PCL) was electrospun and the fibers were mixed with physical hydrogel without session. They both showed fibrous structures fabricated by electrospinning techniques, however, utilizing hydrogel is the major concern in their work. Since physical hydrogel precursors are in liquid state before injection and solidify upon reaching body temperature, after injection, it is diluted with body flow; therefore, mechanical properties designed initially can be changed. Limited diffusion of nutrients for cells is an intrinsic problem for hydrogels. Furthermore, encapsulated cells are rarely placed at the outer layer of the hydrogels; direct cell to the host organ is interrupted.
Therefore, proper techniques to fabricate fibrous structures that allow cells to be distributed not only to the inside but also to the outer layer would be necessary to increase the retention with the hot organ.

In this work, we reported a simple and cost effective technique to generate high aspect ratios of loosely packed fibrous structures via thin polymer film dewetting (Figure 4.5). We chose fully biodegradable and biocompatible PLGA, as a model polymer. Mouse embryonic cells (MEFs) were utilized as a model cell to construct the fibrous structures, and several types of cells were also utilized to verify cell/polymer bundle formation.
Figure 4.5 Two different routes of generation of fibrous cell/polymer constructs. Route 1: cells are seeded directly on imprinted PLGA films. During incubation, thin layers are dewetted and cells are migrated on the PLGA surfaces. Route 2: Fibrous films are generated by dewetting to remove thinner layers. Cells are seeded after transferring the fibrous PLGA sheets on substrates.
4.2.2 Proliferation on Different Surfaces

We first examined proliferation of MEFs on biomaterials in this study. Figure 4.6 summarizes the results of MEFs on surfaces of agarose coated PLGA, and modified PEG compared with TCPS surface as a control. As shown in Figure 4.6a, PLGA surface shows comparable proliferation (absorbance: 0.462 ± 0.015) compared to TCPS surfaces (0.598 ± 0.005) after 18 h incubation. During the incubation, cells were proliferated and spread on the PLGA surfaces (Figure 4.6b), indicating that PLGA showed a minimal negative effect on cell viability and could be utilized for our purpose as it has been verified by others.\textsuperscript{146–148} For the proliferation comparison between bio-fouling surfaces, agarose shows significantly lower absorbance (0.055 ± 0.018) compared to that (0.212 ± 0.014) of PEG modified. It was also verified that few cells were set on the agarose surfaces and the cells were not speared at all (Figure 4.6c). For PEG modified surfaces, unless it has been considered to be the great material for inhibiting protein adsorption and cell adhesion,\textsuperscript{149,150} it did not effectively inhibit cell attachment after 18 h incubation (Figure 4.6d). It is already noted that silanized PEG shows bio-fouling properties within a short-term incubation and looses it at a long-term incubation.\textsuperscript{151–153} After seeding cells on the samples incorporated with PLGA fibrous sheets and agarose coated surfaces, it was found that agarose-coated surfaces effectively inhibited cell adhesion from the surfaces and enhanced the cell adhesion on the polymer surfaces (Figure 4.6e, taken after 18 h incubation of $5 \times 10^4$ cells/mL).
Figure 4.6 Proliferation of MEFs on different surfaces. (e) The absorbance of dissolved formazan formed during the MTT assay of cells attached on the four different surfaces. The optical images of cells attached on (b) flat PLGA films, (c) agarose-coated glass slides, and (d) PEG modified glass slides, (e) PLGA bundles placed on agarose coated glass slides. Statistical analysis by one-way ANOVA ($n = 4$, ***$p < 0.001$). Scale bar: 100 µm.
4.2.3 Stability of Imprinted Films under Cell Culture Condition

The stability of PLGA and PDLA films with and without APTES (3-aminopropyltriethoxysilane, cell adhesion promoter) was examined under the cell culture medium condition. To induce a directional growth of cells on the polymer films, imprinted films with spacing ~ 20 µm of ridges were generated using a featured PDMS stamp (Figure 4.7a). After incubation for 1 day, different phenomena were observed. Dewetting of imprinted PLGA films on glass slides was observed, but only occurred for the thinner strips of the films (Figure 4.7b). Also, the dewetting, was found to only proceed slightly further at a longer incubation time (> 1 day). Dewetting of PLGA films on PEG-silane modified glass slides (Figure 4.7c) and APTES blended PLGA films on glass (Figure 4.7d) were hardly noticed, while cell attachment and spreading on these films were observed. On the other hand, PDLA films (Figure 4.7e), both thinner and thicker strips, were completely dewetted into droplets.

Hydrophobic thin polymer films (< 100 nm) on hydrophilic surfaces are unstable or metastable. If the films have mobility, it undergoes to unstable state, which causes morphological evolutions (e.g. dewetting). At ambient conditions, the destabilizing forces are only van der Waals interactions, however, in aqueous solutions many factors can alter the interactions as shown in Sec. 4.1. For example, proteins in culture medium could adsorb on the films and change the surface properties (e.g., surface energy and zeta potential of the polymers). These effects could diminish van der Waals interactions and electrostatic interactions. Thereby, in aqueous solutions, van der Waals interactions could be utilized for stabilizing forces and electrostatic interactions could be
utilized for desterilizing forces unlike the ambient condition. We found that proteins adsorbed on the PLGA films altered Hamaker’s constant ($A$) across the PLGA films. By assuming mainly adsorbed proteins on PLGA films are bovine serum albumins (BSA), the value of $A$ across the PLGA film decreased from $6.8 \times 10^{-22}$ J (before protein adsorbed on the PLGA films) to $1.3 \times 10^{-22}$ J (Table 4.6). Therefore, van der Waals interactions are weakened but still destabilize the PLGA films. In the case of electrostatic interactions in this system, zeta potential is utilized to estimate whether it can be destabilizing or stabilizing forces. The zeta potential of proteins adsorbed on PLGA was $-14.9 \pm 1.8$ mV (in PBS), which was less negative than that of pure PLGA ($-30.7 \pm 2.1$ mV in PBS) (Table 4.5). It indicates that the reduced electrostatic interactions could lead to the PLGA films less unstable. As a result of reduced van der Waals interactions and electrostatic interactions, dewetting of the films on glass slides under culture medium was suppressed after 1-day incubation. We also examined PEG modified surfaces to induce more cell on the PLGA films (Figure 4.7c). However, the values of $A$ were estimated all negative values before after BSA adsorption on the films (before: $-1.4 \times 10^{-22}$ J, after: $-2.6 \times 10^{-22}$ J, the values are calculated by equation (6) using values reported in Table 4.3 and Table 4.6). In this case, van der Waals interactions act as stabilizing forces for the films. As discussed above, electrostatic interactions became weaken. If it exceeds van der Waals interactions, the films become unstable. According to our observation, hole formation was found but the size did not grow largely after 1-day incubation that could be due to the metastable state of the films. For APTES blended films, suppression of dewetting was observed (Figure 4.7d). This could be possible that
the complicated chain structures of APTES can be entangled with PLGA and amine group can be chemically bonded with hydroxyl groups on glass surfaces.\textsuperscript{158,159}

In the case of PDLA, complete dewetting was observed after 1-day incubation (Figure 4.7e). It could be due to several reasons. PDLA has lower molecular weight ($M_w$) and intrinsic viscosity ($\eta$) ($M_w$: 15 kg/mol, $\eta$: 0.15 – 0.30 dl/g) than those of PLGA ($M_w$: 35 – 60 kg/mol, $\eta$: 0.45 – 0.60 dl/g). Lower intrinsic viscosity facilitates the mobility of polymer. Furthermore, by uptaking water, it can be utilized as a plasticizer, which further enhance dewetting of the PDLA films compared to PLGA films by reducing glass transition of the polymers.\textsuperscript{117,118}

Table 4.6 Values (contact angles of probe liquids) used for estimating surface energy and its components are summarized.

<table>
<thead>
<tr>
<th>Materials/Liquids (mJ/m$^2$)</th>
<th>$\gamma^{tot}$</th>
<th>$\gamma^{lw}$</th>
<th>$\gamma^{AB}$</th>
<th>$\gamma^+$</th>
<th>$\gamma^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA</td>
<td>47.2</td>
<td>43.8</td>
<td>3.5</td>
<td>0.4</td>
<td>7.4</td>
</tr>
<tr>
<td>glass</td>
<td>46.4</td>
<td>43.9</td>
<td>2.5</td>
<td>0.023</td>
<td>65.8</td>
</tr>
<tr>
<td>PEG modified</td>
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<td>45.4</td>
<td>0</td>
<td>0</td>
<td>38.8</td>
</tr>
</tbody>
</table>
Figure 4.7 Generation of imprinted polymer films and different instabilities on several surfaces under cell culture condition. (a) Generation of PLGA imprinted films using a featured PDMS. Stabilities on different surfaces after seeding cells (MEFs, $5.0 \times 10^4$ cells/mL): (b) PDLA films on glass slides, (c) PLGA with APTES on glass slides, (d) PLGA films on glass slides, and (e) PLGA films on PEG modified glass slides. Scale bar: 100 µm.
4.2.4 The Dewetting Process of Imprinting Polymer Thin Films

The topography of imprinted PLGA films was investigated to understand stabilities of the films and to better control the cell/polymer bundle formation. As shown in Figure 4.8a obtained by AFM, it had ~1 \( \mu \text{m} \) height of ridges with spacing ~20 \( \mu \text{m} \). To verify if it had thin layers between the ridges and its thickness, the films were dewetted under DI-water at 50 °C for 15 min. The measured thickness at this layer was 30 ~ 60 nm, which was the thickness range that dewetting occurred (Figure 4.8b).

To understand the stabilities of the thin layer (~60 nm) and the thick layer (~1\( \mu \)m) of imprinted PLGA films, its morphological changes were observed using an optical microscope under 37 °C of DI-water. After incubating for 15 min, the hole formation on thinner regions was observed (Figure 4.8c). Rim formation was observed where holes were initiated after 45 min incubation (Figure 4.8d). After 90 min, rim formations were not observed due to the difference in Laplace pressure that allowed the combination of rims to the thick strips (Figure 4.8e). At this stage, polymer strips were disordered due to the loss of adhesion with substrates by penetrating water at the interface, and formed bundles by entangled with neighbor strips. Earlier suppression of dewetting under the cell medium was not observed under 37 °C of DI-water. The final form of strips was observed to be stable for 14 days at the same condition since interactions through 1 \( \mu \text{m} \) gap (polymer strips) between surrounding and substrates are too far to bounce the polymer layers.
To assure the producing of the cell/polymer bundle formation, we decided to generate fibrous PLGA sheets first by dewetting in DI-water and place it on bio-fouling surfaces to induce more cells to the polymer strips (Route 2 in Figure 4.5). We first utilized PEG modified glass slides, however, cells were spread on the glass slides with a long-term incubation (~3 days) and found cutting was difficult since the surfaces were ridged. Therefore we decided to generate agarose-coated glass slides, which is one of the common non-fouling hydrogels\textsuperscript{78,160}. To prepare sample stage composed of fibrous PLGA films on agarose coated surfaces, the surfaces were prepared by adding 100 µL of 1 wt % of agarose solution in DI-water and dried overnight. Fibrous PLGA sheets were prepared by dewetting as described above and placed on agarose-coated surfaces and two sides of longitudinal direction of fibrous polymer sheets were secured with 5 wt % PLGA solution in acetone (Figure 4.8f).
Figure 4.8 AFM scanned topographies of imprinted PLGA films and progress of dewetting of the films under 37 °C DI-water. (a) The imprinted film has parallel ridges (height: ~1 µm). (b) Thin layer between the ridges has a thickness of 43.7 nm, verified after dewetting. Progress of dewetting at the thin layers is shown in images (c) – (e) obtained by an optical microscope. (c) Holes are formed after 15 min. (d) Holes are combined to form rims after 30 min, and (e) the rims are incorporated to thick ridges after 90 min. (f) Sample stages are prepared by placing fibrous PLGA films on agarose coated glass slides (The size is 1.0 cm × 0.8 cm). Scale bar: 100 µm.
4.2.5 Cell/Polymer Bundle Formation

Cell/polymer bundle formation was observed for 3 days. As shown in Figure 4.9b, cells are distributed all over the surfaces regardless of the surfaces of the polymer and agarose right after seeding cells (5 × 10^4 cells/mL, MEFs). After 4 h incubation, most cells migrated to PLGA strips and few cells were found on agarose surfaces (Figure 4.9c). In this stage, neighbor strips became closer or combined, which was due to the cell attachment and spreading between the strips. After 1 day, it became thick bundles with monolayer of cells by incorporating neighbor strips (Figure 4.9d). The increased number of cells on the polymer surfaces is noticeable, and cell alignment along the direction of polymer strips is observed. After 3 days incubation, multilayer of cells on polymer surfaces were observed and thick cell/polymer bundles were obtained (Figure 4.9e). The radius of cell/polymer was in a range of 60 – 100 µm. In some cases, breakage of bundles (arrow in Figure 4.9f) and secured area were noticed at this point and the broken bundles shrank resulting in thick bundles (D = ~500 µm), attached at the one side of secured area. The breakage could be caused by two effects during the incubation. First, bundles formed by incorporating neighbor fibrous strips and it increases the force across both ends of secured area. At this point, some bundles were broken or the secured area was broken. Second, initially dried agarose gels swell during the incubation and becomes bulged gels, which breaks bundles and secured area.
Figure 4.9 A route of producing cell/polymer bundles from individual polymer strips (a). Progress of bundles formation is shown in (b)-(e). Adjacent fibrous polymer strips are combined during incubation to form the bundles. Right after seeding cells ($1 \times 10^5$ cells/mL) (b), the cells are randomly distributed; 4 h later (c), most of the cells attach on to polymer strips; d) 1 day after incubation, bundles are formed; and e) after 3 days incubation, the outer layer of each polymer bundle is fully covered with cells to form thick bundles ($D \sim 80 \mu m$). If strips are broken (arrow), they retracted and formed thicker bundles ($D \sim 120 \mu m$, f). Scale bar: 100 $\mu m$ for (b)-(e) and 200 $\mu m$ for (f).
Cell/polymer bundles has a high aspect ratio of fibrous structures, which are similar to the blood vessel structure, we examined the bundle formation using rat induced vascular progenitor cells (iVPCs). Monolayer or multilayered cell/polymer bundles (Figure 4.10b and c) were continuously generated similar to cell/polymer bundle formation using MEFs (Figure 4.10a). We further examined bundle formation to verify its clinical usages using other types of cells, including human kidney 293A cell line (Figure 4.10d), human liver cell line (HepG2) (Figure 4.10e) and rat endothelial cells (ECs) (Figure 4.10f). Only ECs did not form the cell/polymer constructs. The exact reasons are currently unclear, but it could be due to the different attachment characteristics of ECs from that of other cell types.

Figure 4.10 The formation of cell/polymer fibrous micro-constructs with different cell types. (a) MEFs, 1 day of incubation, (b) iVPCs, 1 day of incubation, (c) iVPCs, 3 days of incubation, (d) human kidney 293A cells, 3 days of incubation, (e) human liver HepG2 cells, 3 days of incubation, and (f) rat ECs, 3 days of incubation. The seeding cell density was $\sim 1 \times 10^5$ cells/mL. Scale bar: 100 $\mu$m.
4.2.6 Summary

We generated cell/polymer bundles, which were mainly composed of fibrous PLGA polymers and cells grew in and outside of the bundles, to mimic the fibrous tissue structures. We used thin polymer dewetting, which is a simple and cost effective method, to remove thin-layer of imprinted patterns and to generate loosely packed fibrous polymeric structures. Due to the different instabilities on directly imprinted PLGA films on silanized glass slides, we used agarose-coated glass slides, which effectively inhibited cell adhesion more than 7 days. As a result, the agarose-coated surfaces induced cell attachment only on the PLGA surfaces. While cell-polymer bundles were prepared by seeding MEFs, iVPCs, human kidney 293A cells, and human liver HepG2 cells, bundle formation was not achieved by seeding ECs. During 3 days incubation, cells were grown in and outside of the bundles since the fibrous polymeric supports were loosely packed. Considering its highly porous structures and a high coverage of cells on outer layers of the constructs, it could be considered to be an effective cell delivery vehicle.
4.3 Cell/Polymer Micro Bundles as a Noble Injectable Scaffold to Enhance Cell Viability and Retention

This part demonstrates the results and discussions about cell/polymer micro bundles as a noble injectable scaffold to enhance cell viability and retention. The subsections include introduction, morphological observation and injectability of bundles, effect of injectability on the geometries of bundles, retention study, viability study, enhanced viability of post injected cells \textit{in vitro}, effect of polymer strips on the increased viability, cell migration towards the polymer strips, injectability of cell/polymer fibrous micro constructs \textit{in vivo}, and summary.

4.3.1 Introduction

Direct injection is clinically preferred due to its easiness and invasive natures.\textsuperscript{161–165} Two most common direct injections are injection of free cell suspension and injection of cells encapsulated with hydrogels. These approaches in cell therapy have shown potentials for improving tissue and organ functions for various diseases including peripheral arterial disease,\textsuperscript{166} bone diseases,\textsuperscript{167} myocardial infarctions,\textsuperscript{164} and parkinson’s disease\textsuperscript{169}. However, the approach exhibits many shortcomings that have not been resolved.

For free cell injection, low retention at the target site has been reported.\textsuperscript{168,170} Major causes of the low retention ratio have been noticed as the hypoxic microenvironment and an acute immune response by the host organ.\textsuperscript{171} Besides the issues associated with host organs, a recent study revealed that a significant cell death
(~40 %) could be resulted from the extensional flow from a syringe barrier to the narrow needle (G 28) during injection. To increase cell viability during injection, shear-thin delivery using hydrogels has been utilized. Hydrogels are believed to be effective carriers with their porous nature and biodegradability, some clinical potentials have been noted. Unfortunately, lack of long-range micro architectural channels, low and inhomogeneous mechanical strength, and sterilization issue are some documented shortcomings for hydrogels. Moreover, hydrogels interrupt the direct contact of cells with the host organ, which can cause low incorporation of injected cells to the host organ. Tunable mechanical properties and degradation nature are considered to be the major merit for hydrogels; however, proper degradation with the growth of encapsulated cells has hardly been achieved since synthetic polymers show the bulk degradation and the mechanical strength and the degradation rate are inversely related. In addition, injectable hydrogels could not promote directional growth of implanted cells since it has no inner tracks to guide the growth.

In this study, we proposed the development of a new type of scaffolds containing cells with micro fibers (cell/polymer micro-bundles) and demonstrated that it could be an effective injection based cell delivery vehicle. Figure 4.11 shows the schematic of our approach. The studies found that three-day incubation was optimal for forming injectable micro bundles, and a large passage rate resulted with bundles having length of 10 mm or less. Using a tapered syringe barrel, the injectability increased almost twice as compared to using the conventional syringe barrel. Furthermore, the injected cell/polymer bundles retained on the target sites with enhanced viability and proliferation after incubating for
14 days post injection compared to free cell injection. In addition, *in vitro* retention study was confirmed from preliminary *in vivo* study.

Figure 4.11 Schematic illustration of bundle formation and subsequent bundle injection: (a) Cells are seeded on parallel strips of PLGA that is placed on a glass slide coated with a thin layer of agarose gel. (b) The cells proliferate on the polymer strips. (c) Bundles are sectioned into smaller pieces, and then (d) delivered into the target place by an injection with a G 20 needle.
4.3.2 Morphological Observation and Injectability of Bundles

Injection feasibility was first examined using 1 – 3 days incubated cell/polymer bundles. Two types of injections, conventional injection (Design A) and our design (Design B), were used to assess the injectability (Figure 4.12a and b). The cell/polymer bundles were cut into ~1 mm length and ~ 30 bundles were utilized for each case. Injectabilities were verified by counting ejected bundles from ~ 30 bundles with 500 µL of cell culture medium inserted in a syringe. Injectabilities were shown increasing trends from 10.3 ± 7.0 % to 39.5 ± 8.7 % for Design A and 35.6 ± 5.1 to 75.2 ± 5.6 % for Design B (Figure 4.12c). Overall injectability of Design B showed significantly higher than that of Design A regardless of incubation days. For example, the injectability of 1 day incubated bundles using Design A was improved to ~190 % (at 2nd day) and ~210 % (3rd day) by incubating 1 day more. Compared to Design A, injectabilities of bundles incubated 1 – 3 days using Design B were dramatically increased to 346 % (at 1st day), 300 % (2nd day), and 190 % (3rd day). The overall increasing trends along the increasing incubation time for both types of injection should be related to the cell coverage on the polymer surfaces. Cells grew in and out side of bundles during 1 day incubation, however did not reach to a full coverage (Figure 4.12d1). By prolonged incubation, increased cell coverage is noticeable (Figure 4.12d2 and 4.12d3). It is reported that surface tension of cells is ~68 mJ/m² and our measured surface tension of cell culture is ~68 mJ/m². Considering surface tension of PLGA ~47 mJ/m², increasing cell coverage decreases the interfacial tension differences between culture medium and cell/polymer bundles, which reduces hydrophobic interactions with other bundles. In this reason, bundles can be distributed throughout in culture medium, and follow the flow of the fluid.
In addition, cells act as glue to combine polymer strips within the bundle structures to maintain the initial structure against flow dynamics during injection.

Figure 4.12 Injectabilities of bundles are assessed using two different injection designs. (a) A conventional syringe/needle design, and (b) replacing the conventional syringe barrel with a tapered pipette tip. (c) The comparison of injectability of bundles with different incubation times using the two designs of injection. The images of bundles before (d) and after (e) injection for bundles formed after 1(1), 2 (2), and 3(3) days of incubation. The florescent images (far right) of the bundles with 3 days of incubation presented are also presented. As shown in the florescent images, cell death caused by injection is not significant. Significantly different groups are indicated by letter using two-way ANOVA, where “A” represents the highest mean (n = 30, p < 0.001). Scale bars: 100 µm.
The low injectability using Design A is due to the different diameters from the tubular syringe luer lock tip to entrance of a needle. When the needle is locked into the syringe, the luer lock tip does not push all the way down against the entrance of the needle. The spacing between the luer lock tip and the entrance of the needle could create a stagnant zone and some of the bundles could be stuck during injection. At this zone, the broken bundles increased its overall diameters or had higher chances to entangle together with other bundles, therefore resulted in low injectability. The conventional injection is designed to deliver liquid drugs and not specified to deliver injectable scaffolds. For effective delivery of injectable scaffolds, simple designs would be better. Therefore, we developed the design of injection. For Design B, by cutting ~ 1 cm of 1 mL micropipette, it fitted tightly with the needle valve and closely attached to the entrance of the needle. Since it has gradually decreased diameters, directional flow is more favorable than Design A and resulted in higher injectability.

Unless Design B showed promising injectability, 1 day incubated bundles would not be utilized for injectable carriers. As shown in Figure 4.12e1, bundles did not maintains its initial shape (Figure 4.12d1) and massive cell detachment was found when it was cut into smaller pieces and after ejection. Although some of bundles can be injected in the host organs, the exposure of polymer surfaces will decrease the engraftment ratio with host organ. In the case of 3-days incubated bundles did not show large difference in its original shape before (Figure 4.12d3) and after injection (Figure 4.12e3). Detached cells were rarely found after injection and the majority of cells after injection showed alive (green florescent images). The outer cell layer will directly
contact with the host organ. Therefore, 3 days incubated bundles and Design B were utilized for further studies.

4.3.3 Effect of Geometries of Bundles on Injectability

The series of injectability depending on the length scale of bundles were evaluated using Design B injection. In this study three different lengths (0.5 mm, 1.0 mm, and 2.0 mm) of bundles (diameters: 60 ~ 100 µm). As shown in Figure 4.13, the injectabilies were inversely proportional to the length of the bundles. The shortest showed highest injectability of 76.8 ± 10.8 % and the longest showed the lowest injectability of 61.7 ± 6.5 %). The injectability of ~1 mm length of bundles was only ~1 % lower (75.2 ± 9.0 %) compared to that of the shortest. It was found that longest bundles were not easily distributed in cell culture medium, which could be one of the reasons of low injectability. Before ejecting the bundles, bundle contained cell medium in an injection was gently tapped to distribute them before injection. The bundles of 0.5 mm and 1.0 mm were easily distributed but the longest bundles (2.0 mm) were found to be entangled together with neighbor bundles and not separated easily once they contacted. From these injectability studies, we found that optimum conditions for promising injectability were 3 days incubated bundles with length of ~1 mm. We utilized bundles from these conditions for further evaluations.
Figure 4.13 Injectability of bundles with different aspect ratios. The percentage pass rate (~76%) of bundles (3 days incubated) with a length of ~0.5 mm (b) and ~1 mm (c) are similar, but it is decreased (to ~60%) using bundles with a length of ~2 mm (d). The diameter of the bundles are ~100 µm. Scale bar: 100 µm. Statistical analysis by ANOVA ($n = 30$; *$p < 0.05$).
4.3.4 Retention Study

Retention of cell/polymer bundles for 7 days incubation was examined using an alginate gel as an *in vitro* environment. Gels were prepared by adding 3 mL of 1 wt % sodium alginate gel solution (in cell culture medium) in 3 mL of 1 wt % calcium chloride solution in PBS. Crosslinking was achieved by replacing Na\(^+\) to Ca\(^{2+}\) ions and to produce soft gels, we stopped the crosslinking within 2 min by removing unreacted solutions and rinsing with PBS thoroughly. After removing PBS and adding 3 mL of cell culture medium, bundles were injected to the center position of the gels as shown in Figure 4.14a. After 3 – 7 day incubation, it was dissolved by adding of 10 wt % citrate acid solution in PBS to assess the viability of bundles (Figure 4.14b). Enlarged image (Figure 4.14c) shows initial retention of bundles in a gel. After incubating 7-day, it was found that the bundles were retained where they were initially delivered (Figure 4.14d). Interestingly, cell proliferation was also observed compared to two enlarged images of initial (Figure 4.14e1 and f1) and incubation for 7 days (Figure 4.14e2 and f2). It showed that the cell layers became thicker during incubation time and found still alive (Figure 4.14e3 and f3).
Figure 4.14 Retention evaluation of cell/polymer bundle injected in vitro. (a) An in vitro environment made of sodium/calcium alginate gel containing injected cell/polymer bundles. (b) The dissolved state of the gel using 10 wt % sodium citrate after 7 days incubation. Enlarged images show bundles right after injection in the gel (c), and after 7 days incubation (d). Cell coverage on the bundles became thicker after 7 days of incubation (e2 and f2) as compared to its initial state (e1 and f1). The corresponding fluorescent images (e3 and f3) of (e2) and (f2) show that most cells are alive. Scale bar: 500 µm for (c) and (d), and 200 µm for (e) and (f).
To compare the results of the observation from cell/polymer bundle injection, free cell injection was also studied. In our first attempt to observe the retention of cells injected, the same places initially imaged could not be easily found during the incubation time since the size of cells were small (~15 µm), therefore, ~ 5 bundles as a marker were delivered together with cells at the center location of the gels. The cell/polymer bundles were retained at the initial locations, however, most cells were found at the top inside of the gels (Figure 4.15a). It was found that most cells were found at the top inside of the gels regardless of the places where cells were delivered (center or bottom of the gels). We speculated that it could be due to the dilution of gel precursor solution not fully crosslinked by injection of cell suspension. When cell suspension is injected, it could dilute the gel precursor solution inside of the gel and pressurized the gel. The only place where the pressure can be released is a needle-inserted area, therefore, the cells with the medium was leaked or overflowed into the direction of the needle inserted. According to such effects, cells were found at top (Figure 4.15b2) and none of cells were found at bottom (Figure 4.15b4) of the gel inside. The cells injected were floated right after injection and the cells were remained at the top layer and did not sink down during the incubation time (~7 days). In the case of bundles injection, leakage of cell culture medium was observed, however bundles did not leak out or float to the top layer of the gels. After 7-day incubation, the cell distributions at the top, middle, and bottom of the gels were the same with its initial state (Figure 4.15c2 – c4). It was found that initially round shape of individual cells formed large size of clusters by incorporating with neighbor cells. In addition, some cells initially contacted with cell/polymer bundles were incorporated to adjacent bundles (arrows in Figure 4.15b5), however, cells far from the
bundles did not show migration to the bundles. From these retention studies using an alginate gel environment, we believed that cell/polymer bundle could be a potential cell carrier, which would result in better retention compared to free cell injection.

Figure 4.15 Retention of dissociated cells in an alginate gels as an in vitro environment. Several bundles were also delivered in alginate gels as position markers to find the initially injected site. (a) A schematic representation of the locations imaged. (b) Right after injection and (c) after 7 days of incubation. The bundles were retained their initial locations (1). Individual cells became cell clusters at the top (2) and the middle (3) and no cells were found at the bottom. Near the bundles, individual cells (indicated by arrows in b5 and b6) could no longer be seen (c5 and c6) and they clustered and migrated to the bundles after incubation. Scare bar: 500 \( \mu \text{m} \) for (b1) and (c1), 200 \( \mu \text{m} \) for (b2–6) and (c2–6).
4.3.5 Viability Study

Viabilities and proliferation of cell/polymer bundle injection in an alginate gel were assessed for 14 days as well as those of free cell injection as a control. Prior to assess the viabilities, we examined the effect of 10 wt % sodium citrate acid solution on the cell viability during the dissolution of the gel. $3 \times 10^5$ cells dispersed in 500 $\mu$L of cell culture medium was injected in an alginate gel using G 20 needle, then dissolved in 10 mL of 10 wt % sodium citrate acid solution. The obtained viability was $93.2 \pm 1.2 \%$, which was similar to the viability of free cells after injection, which was $92.8 \pm 2.8 \%$. Therefore, it has little to no effect on viability.

Next, we tested viabilities of before and after injection of cell/polymer bundles and compared to those of free cell injection. To equivalent the number ($3 \times 10^5$ cells) of cells using bundle or free cell injection, we first counted number of cells on bundles using trypan blue exclusion method. It was found that $357 \pm 22$ cell/polymer bundles with ~1 mm length obtained by from 8.5 sheets (3 days incubated) was equivalent to $3 \times 10^5$ cells. Figure 4.16 summarizes viabilities before and after injection. Before injection, viability of free cells was $97.6 \pm 0.9 \%$ and it was decreased to $92.8 \pm 0.9 \%$ after injection. Since free cells did not have structural integrity, extensional flow and shear forces broke the cell membrane resulted in ~ 5 % decreased viability. The reduced viability was in a good agreement with others.\textsuperscript{51} In their study, reduced viability was ~42 \%, which was significantly higher than our result mainly due to utilizing larger gage ($D = \sim 200 \ \mu$m) than ours ($D = \sim 600 \ \mu$m). For bundle injection, initial viability was measured to $88.0 \pm 2.8 \%$, which was ~9 % lower than that of free cell injection. However, similar viability
was measured to $87.7 \pm 2.2 \%$ for after injection, indicating that structural integrity of cell/polymer bundle provided protective effect during injection.

Figure 4.16 Cell viability before and immediately after injection, assessed by trypan blue. The viability of free cells (dark grey bars) before and immediately after injection was $97.6 \pm 0.9 \%$ and $92.8 \pm 0.9 \%$, respectively. For bundles (lighter grey bars), the viability before injection was $88.0 \pm 2.8 \%$, and it was $87.7 \pm 2.2 \%$ immediately after injection. Significantly different groups are indicated by letter using two-way ANOVA, where “A” represents the highest mean ($p < 0.001$).
After confirming protective effect from the structural integrity, we conducted separate study to assess the viability and proliferation of cell/polymer bundles for 14 days. To deliver the same number of cells \((3 \times 10^5)\) by free cell injection, \(~470\) bundles were injected in alginate gels. To inhibit the leakage during injection and incubation, the needle inserted area was secured by adding \(\text{CaCl}_2\) solution. As shown in Figure 4.17a, initial viability of free cell injection in alginate gels was \(92.3 \pm 2.5\%\) and continuously decreased to \(67.4 \pm 3.0\%\) at 14 day. Initial viability of bundle injection was \(89.3 \pm 4.5\%\) and decreased to \(79.5 \pm 1.2\%\) at 14 days. For bundle injection, initial viability \((89.3 \pm 4.5\%)\) was \(~7\%\) lower than that of free cell injection, however, at 3-day incubation, the viability was \(90.5 \pm 1.9\%\), which was the similar viabilities of the injection of bundles before and free cell injection incubated for 3 days. Continued viability study showed that both cases showed decreasing trend, however, viability of cell suspension injection showed more rapid decreasing trend compared to that of bundle injection. For example, % decrease viability for 14 days injection was \(24.9\%\) for free cell injection and \(9.8\%\) for bundle injection, resulted in \(~18\%\) higher survival rate of cells on the bundles than free cells inside the alginate gels. More importantly, as shown in Figure 4.17b, significantly higher proliferation \((~47\%\)\) on bundle injection compared to free cell injection was found at 14-day incubation. Around 7 days incubation, the numbers of cells for both cases were doubled, however, only bundle injection maintained similar proliferation rate at additional 7 day incubation (at 14 day incubation).

One of the reasons for decreasing trend of viability for 14-day incubation is due to the limited diffusion of hydrogels. Improper supply of nutrients and oxygen for cells
causes reduced viability during incubation times for both cases. Another reason for higher viabilities of bundle injection than those of free cell injection at 7 and 14 days are due to the high aspect ratio of fibrous structures of cell/polymer bundles. While proliferated cells are evenly distributed on the bundles during the incubation, freely delivered cells construct cell clusters. Such spherical shapes of cell clusters became larger during incubation and it limits diffusion of nutrients to cells inside of the clusters.

One might question about the diffusion issue since locations of bundles and dissociated cells were different. Bundles were retained at the middle position of alginate gels where they were delivered while dissociated cells were floated from the middle position to the top inside of the gels. It has been reported that it has similar diffusion efficiency compared to collagen when its $M_w$ is lower than 500 kD. Lan et al. utilized 3D alginate disk (diameter × thickness: 15 mm × 700 μm) as an in vitro co-culture model platform for the toxicity screening of chemicals. In their study, a density of $10^7$ cells/mL was encapsulated in the disks and its viability for 3 days was ~80%. According to optically sectioned images, dead cells were uniformly distributed throughout the sectioned images indicated that the diffusion did not largely affect on cell death.

In our study, 3 days incubated bundles and free cells in alginate gels showed viability of ~90 % for both cases, considering our gels are significantly larger (~7mL) than their gels and cell culture medium (~70 v %) was mainly contained in the gels, it could be concluded that diffusion did not significantly affect on cell death in our in vitro environment.
Figure 4.17 Viability (a) and proliferation (b) of MEFs in an alginate gel environment for 14 days. Viability and proliferation data are obtained by counting live/dead cells with aid of a hemocytometer after staining the cells with trypan blue at each day. Significantly different groups are indicated by letter using two-way ANOVA, where “A” represents the highest mean ($n = 3, p < 0.001$).
4.3.6 Enhanced Viability of Post Injected Cells in vitro

We revealed that bundles showed higher viability and proliferation compared to free cell injection. In this section, we demonstrated which factors enhanced the viability and proliferation for bundles.

4.3.6.1 Effect of Polymer Strips on the Increased Viability

Viability of free cells injected in alginate gels contained PLGA strips was examined to reveal the effect of polymeric structures on the increased viability of cell/polymer bundle injection. As discussed in earlier sections, cell/polymer bundles showed the increased viability and retention in vitro compared to direct injection of cell suspension. Since polymers have a hydrophobic nature, it is hardly delivered with aqueous solutions by injection. Therefore, alginate gels contained fibrous polymer sheets were first prepared, and cells were injected to assess the viability of free cell injection. As a control, alginate gels without polymers were prepared for free cell injection.

Two locations inside of the gel were observed in this study for 7 days: Location 1 – the cells were injected near the fibrous sheet and location 2 – cells were not delivered (Figure 4.18). Compared to right after injection and after incubation for 3 and 7 days, significant morphological changes were not observed both at the location 1 and location 2. At location 1 (normally top inside of the gels) where cells were initially attached, the morphology changes during incubation time were difficult to distinguish if any increase of cells at these places occurred. This might be due to the difficulties of observing under aqueous solutions with randomly distributed fibrous polymer sheets. At location 2
(normally, bottom inside of the gels), initially cells were not attached and any of cell attachment was not observed for 7 days.

Figure 4.18 Cell attachments on PLGA fibrous sheets at different locations during 7 days incubation in alginate gels: cells are initially attached on the polymer sheets (location 1) and cells are not initially attached on them (location 2). Scale bar: 100 µm.
For the clear observation of cell proliferation on polymer bundles, one of gels, after 7 days of incubation, was dissolved in a sodium citrate solution (10 wt % in DI-water) and the morphologies were imaged (Figure 4.19a and b). In this case, cell attachment on polymer strips was clearly noticed. Proliferation due to the PLGA films could not be verified by observation using an optical microscope. The other methods to determine the viability, such as staining cells with trypan blue, were applied (Figure 4.19c). According to a separated control experiment of injecting free cells into the gel without the PLGA film, the viability of free cells injected after 7 days of incubation inside the gel was 61.2 ± 3.5 %, which was slightly higher than that (51.0 ± 4.6 %) of earlier studies (in Section 4.3.5.). The viability of cells injected to the gel containing with the PLGA sheet was 75.9 ± 2.9 %, which was slightly lower as compared to that (77.8 ± 6.6 %) of the earlier studies of the injected cell/polymer bundles. The morphological evidences of cell proliferation on the PLGA films were not found from this study. However, it was clear that free cell injection in the presence of PLGA film increased cell viability up to 124 % compared to that in the absence of PLGA film.
Figure 4.19 (a) and (b) different focuses for representative images of cell attachment on polymer strips after dissolving an alginate gel. (c) Viability of free cell injection in an alginate gel with and without fibrous PLGA sheets in an alginate gel after 7 days incubation. Statistical analysis by one-way ANOVA ($n = 3$, $***p < 0.001$). Scale bar: 100 $\mu$m.
4.3.6.2 Cell Migration Towards the Polymer Strips

Figure 4.20 presents cell migration within 30 min after seeding cells ($5 \times 10^4$ cells/mL) on a sample stage. Cells placed on the agarose-coated surfaces initially had round shape and the protrusion of cell cytoskeleton was noticed as the cell was trying to find an anchor to the surfaces (Figure 4.20a). It appeared cells have a preference of the PLGA bundles more than the agarose surface, and cells liked other cells even more, as cells initially attached to the polymer bundles migrated to other cells. The arrows indicate cell migration direction (Figure 4.20b) and the circles indicate cell aggregation (Figure 4.20c).

Figure 4.20 Cell migration towards to the PLGA strips during cell/polymer bundle formation. (a) Randomly distributed cells right after seeding cells ($5 \times 10^4$ cells/mL). (b) Enlarged image of (a). (c) Cell migration 30 min after seeding cells. Scale bar: 100 µm for (a) and 25µm for (b) and (c).
To confirm the migration of cells to PLGA bundles from the agarose-coated surfaces, additional study was performed. As shown in Figure 4.21, substrates composed of two different surfaces of agarose and PLGA were prepared. Right after seeding cells (\(5 \times 10^4\) cells/mL), cytoskeleton protrusions (arrows in Figure 4.21a) were noticed as cells seeking a desire surface to attach. 1 h later, most of cells had migrated to be segregated close to the polymer films and to each other, no cells were found on agarose-coated surfaces (Figure 4.21d). On the polymer bundles, the shape of cells initially round had changed, which might be as a process for further spreading. Therefore, after 4 h, no cells were found at where initially cells were seeded on the agarose-coated surfaces as shown in Figure 4.22. The enlarged images show cell attachment on to bundles and cell alignment through the bundles (arrow in Figure 4.22c).

In addition, this study explains why free cell injection in alginate gels showed viability up to 51 % after 7 days incubation. Cells need to attach to surfaces where they can survive and proliferate. Neighbor cells could migrate to form cell clusters to increase the viability since they could not found surfaces to attach inside of the gel. In addition, it explains higher viability of free cell injection in the presence of PLGA strips as discussed in Sec. 4.3.5.
Figure 4.21 Cell migration from agarose surfaces to PLGA strips within the first hour after seeding the cells. Images were taken (a) right after seeding cells ($5 \times 10^4$ cells/mL), (b) after 15 min, (c) 40 min, and (d) 1 h. Scale bar: 25 $\mu$m.

Figure 4.22 Cell attachments on PLGA strips and no cell attachment on agarose surfaces after 4 h incubation (a). Its enlarged two boxed images of 1 for (b) and 2 for (c). Scale bar: 100 $\mu$m for (a) and 25 $\mu$m for (b) and (c).
4.3.7 Injectability of Cell/Polymer Fibrous Micro Constructs *in vivo*

We showed that cell/polymer bundle could be a potential candidate for injectable cell delivery constructs *in vitro*. To demonstrate the potential of the injectable cell/polymer fibrous micro-constructs in cell-based therapy by injection, the micro-constructs containing iVPCs or free iVPCs were injected into myocardium of SD rats. First, we injected free iVPCs (stained by Td-red) or iVPCs/PLGA micro-constructs mixed with microspheres (labeled with FITC) into normal rat heart to check for cell survival and cell retention. Figure 4.23a and b shows fluorescent images of sections of left ventricle stained with DAPI (blue) after injection of free iVPCs (Figure 4.23a) or iVPC/PLGA micro-constructs (Figure 4.23b). More iVPCs aligned with the micro-constructs, suggesting iVPC/PLGA micro-constructs resulted in a better cell retention and/or cell survival than free iVPCs. A rough estimate by counting cells using ~ 10 images showed that at least 10 X cells, as compared to injected free cells, retained at the injected site when injected with constructs. The better survival and retention of these cells suggested that the fibrous micro-constructs improved cell survival/retention after injection. Also, the loose scaffolds and cells located on these loose scaffolds would more likely allow the cells to interact better with the microenvironment than cells being encapsulated, leading to increased survival. More importantly, as the polymer scaffolds degrade at a later time point, the cells could migrate to where they needed to be with the cue of tissue repairing.
Figure 4.23 The representative images of injection results *in vivo*. Injection of (a) free iVPCs and (b) iVPCs/PLGA constructs in rat hearts four weeks after the injection therapy. (c) and (d) are the enlarged sections boxed in (a), (b) respectively. iVPCs were labeled with tdTomato (red) and the nuclei of other cells were stained by DAPI (blue). Scale bar: 100 µm.
4.3.8 Summary

In this study, cell/polymer micro-bundles for injection based cell therapy was examined with respect to injectability (before and after injection), viabilities (before and after injection and 14 day incubation \textit{in vitro}), and retention in an \textit{in vitro} model. We briefly showed the formation of the cell/polymer micro-bundles during incubation days. Optimum conditions for effective delivery using a G 20 needle were found at the length up to 1 mm of bundles incubated 3 days with a seeding density of $1 \times 10^5$ cells/mL. Comparison between free cells and the bundles showed that the bundle injection showed the effective delivery after ejecting from the G 20 needle and higher viability and proliferation after 14 day incubation \textit{in vitro} than that of free cell injection. Our retention study also showed that the bundles delivered in an alginate gel were left at the injected places during 7 day incubation while cells were floated at the top of the gel from the injected site right after the delivery. More importantly, cell retention \textit{in vivo} was significantly higher for bundles injection compared to free cell injection. From our results, it is concluded that the cell/polymer micro bundle injection showed the effective protection of cells during injection and great retention after injection. Compared to current approaches, our noble approach has a great potential for cell delivery of tissue engineering and regenerative medicine.
4.4 Fully Biodegradable Injectable Tubes via Self-Rolling Bilayer Films for Cardiovascular Disease

This part demonstrates the results and discussions about fully biodegradable injectable tubes via self-rolling bilayer films for cardiovascular disease. The subsections include introduction, tube formation via self-rolled bilayer films in cell culture conditions, capability of cell encapsulation in tubes, aspect ratio effect on tube shapes, inner patterned structures in tube Injection feasibility of tube, co-culture of SMC and ECs in patterned bilayer films, and summary.

4.4.1 Introduction

Cardiovascularization is an important topic in cardiac disease for supplying nutrients and oxygen to replace damaged blood vessels. Injection of stem cell suspensions is routinely utilized due to its simple and minimally invasive method. The method expects that the stem cells delivered into the defected site can differentiate into blood cells and self construct blood vessels. Major issues that come forth from this approach is low retention of cells at the host organ\textsuperscript{12,50} and cell loss during injection.\textsuperscript{51,52} Due to these reasons, scaffolding systems have been actively studied for precise localization and increased cell survival by attaching cells to the surface of scaffolds or encapsulation of cells in scaffolds.\textsuperscript{180–182} It might be a promising approach, however, it would be better to incorporate anisotropic structures in scaffold design to achieve truly mimic the native tissue structures. Blood vessels have a long tubular structure, which are mainly composed of endothelial cells (ECs) and smooth muscle cells (SMCs). ECs layer
is the innermost layer, which contacts with blood, and SMCs layer is the outermost layer that inhibits the leakage of blood and supports the structures.\textsuperscript{183,184}

To mimic the native blood vessels, high aspect ratios of tubular structures are more highly favored. SMCs should grow in helical direction and cover the ECs layer, which is found in native tissue. To achieve such structures, self-folding bilayer films could be the best choice. It has unique properties, for example, initial 2-D films can be formed into ordered 3-D structures by external stimuli.\textsuperscript{185,186} In addition, the actuation can be controlled with many different valuables such as, thicknesses of bilayers and aspect ratios of patterns. Currently, several types of self-rolling bilayer films are generated by thermal control and water uptaking control. Thermal control has bilayers of active and passive layer with different thermal expansion rates (passive layer placed on active layer).\textsuperscript{188} By increasing the temperature, the active layer expands but the passive layer inhibits the expansion of the active layer. When the passive layer losses the ability to inhibit, the active layer bends. Unfortunately, the bilayers are composed of non-biodegradable inorganic materials. For water uptaking approach, the bilayer films are composed of hydrogel (active layer) and biodegradable polymer (passive layer) such as polycaprolactone.\textsuperscript{112,113} Having the same phenomenon, the active layer expands and the passive layer induces the ordered active layer rolling, but in physiological condition. Since hydrogels (for active layers) and biodegradable polymers (for passive layers) have a variety of selections and routinely utilized in biology, it could be a powerful approach for injectable cell carriers. Stroganov et al. developed hydrogel based self-rolling bilayer films.\textsuperscript{113} They utilized it for nerve generation since tubular structures are alike to the
native nerve tissue and showed feasibility of encapsulation of neuron cells. Major concern for utilizing this method is the use of hydrogel. For instance, to achieve a tube with a diameter around $10 \rightarrow 20 \mu m$, $100 \text{nm}$ thick poly($\varepsilon$-carp)rolactone (PCL) and $1.7 \mu m$ thick gelatin layer were needed. The thick wall of the tubes resulted from rolling of the bilayer films would limit the transportation of nutrients and oxygen for cells for long-term in vivo. Another concern is the generation of helical structure of SMCs. In their work, photolithographic technique is utilized for fabricating rectangular bilayer patterns, therefore, inner patterning on bilayer film patterns needed complicate several steps using expansive photo masks.

In this study, an alternative and simple approach to generate self-rolling bilayer films to construct 3-D injectable tubular structures for delivering cells was tested and proved to be efficient. The bilayer films are composed of biocompatible and biodegradable polymers, PDLA for adhesive layer and PLGA for active layer, which have been widely utilized for fabricating scaffolds. The programed bending on PLGA films are placed on PDLA layer (Figure 4.24a). By incubating the bilayer films in cell culture conditions, PLGA layers undergo rolling due to the stress relaxation when PDLA layer loses the adhesion from the substrates by dewetting (Figure 4.24b). Since PLGA films are generated via soft lithography, pattern generation on PLGA films is very simple. We demonstrated that mechanism of self-rolling of bilayer films with respect to different initial strains. For the biological application, we tested the feasibility of whether the tube could be an injectable carrier including encapsulation capability, injectability, and
viabilities. We also attempted to fabricate pre-formed blood vessels using bilayer films, which might enhance the tissue regeneration.

Figure 4.24 Schematic of cell encapsulation via self-rolling of bilayer films.
4.4.2 Tube Formation via Self-rolling Bilayer Films in Cell Culture Condition

Bilayer films with high aspect ratios (8:1) of PLGA (active layer) and PDLA films (passive layer) on glass substrates were examined to generate tubular structures. 600 ± 125 nm thick PLGA films were fabricated by pressing 20 µL of 9 wt % PLGA solution in acetone in between flat PDMS pads. After releasing one of the pads, the films were placed on 330 ± 9 nm thick PDLA films on glass slides (1 cm × 1 cm). The bilayer films became tubes by rolling from both sides (long-side rolling) after 48 h incubation in 37 °C cell culture medium. As shown in Figure 4.25, the films were stable in the medium initially (Figure 4.25a) and instability was observed after 1 h (Figure 4.25b). After 4 h, bilayer films started diagonal-rolling from all edges (Figure 4.25c), but it was switched to long-side rolling at 12 h (Figure 4.25d). After 48 h, bilayer films became tubes via long-side rolling (Figure 4.25e). The final structures were composed of tubes with two small diameters made by rolling from two long-sides (Figure 4.25f). Our observation agrees with the other studies.\textsuperscript{189,190} All the edges of the films were experiencing the first rolling phenomena since the four edges have the highest possibility of water uptake or water penetration through the interface of PDLA-substrate followed by long-side bending due to the bending force being greater than that of short ones.

In our case, the PDLA layer confined with the PLGA layer acts as a passive layer for controlled bending in one direction and a temporal adhesive layer for controlled detachment of bilayer films from a substrate. In the role of the passive layer (i.e., PDLA layer), it causes strain gradients to z-direction of the PLGA layer if the PDLA layer has zero strain or a smaller strain than that of the PLGA layer (Figure 4.26a). Therefore, the
bending direction is perpendicular to the active layer, which results in rolling of bilayer films. It is similar to the role of a passive layer on hydrogel based self-rolling of bilayer films, which have a design of an active layer (dried hydrogels) on substrate confined by a passive layer (e.g., poly(ε-carprolactone)) (Figure 4.26b).\textsuperscript{112,113} Swelling of the active layer occurs from all sides of the bilayer patterns since water penetration from z-direction is inhibited by the passive layer. When the hydrogels swell and experience the large volume expansion, the passive layer inhibits the expansion of x- and y-direction. As a result, the active layer bends and rolls to the perpendicular direction against the passive layer. Such phenomenon explains our preliminary studies using PLGA films on glass surfaces without PDLA films (i.e., passive layer) and PLGA films on 150 nm thick sucrose-coated glass surfaces (data not shown). For both cases, bending or rolling of PLGA films (generated by the same method in this study) immersed in cell culture medium was not achieved. Instead, they were deformed without any uniform shapes after detaching from the substrates since strain gradients on PLGA films cannot be achieved without utilizing the passive layer.
Figure 4.25 3-D tube formation via rolling of bilayer films. (a) Right after adding cell culture medium to the bilayer films (pattern 4 mm × 500 µm); (b) after 1 h, instabilities were seen in the films; (c) after 4 h, rolling from edges was visible; (d) after 12 h, most rolling occurred along (parallel to) the long sides; (e) after 48 h, tubes formed. (f) SEM images show the resulting tubes formed by rolling from two long edges of a bilayer. The thicknesses of bilayer films were 600 nm and 330 nm, respectively for PLGA and PDLA films. Scale bar: 400 µm for (a) – (d) and 100 µm for (e) – (f).
Figure 4.26 Comparison between (a) our approach and (b) hydrogel based self-rolling bilayer films. For both cases, the passive layers induce strain gradient on the active layers. In our cases, the passive layers also act as a temporal adhesive.
The main difference between our method and the hydrogel based approach is the detachment mechanism. In our case, the passive layer (i.e., adhesive layer) located underneath the active layer acts as a temporal adhesive that controls the rolling direction of the active layer by detaching from the substrate. By absorbing a small amount of water into the adhesive layer, it is utilized as a plasticizer and mobility is obtained by reducing its glass transition temperature. At this point, thin polymer dewetting is exerted and adhesion to substrate is reduced unless it is confined by the active layer. According to Lin et al., bilayer films undergo dewetting when the viscosity of the confined layer (in our case, passive layer) is lower than the upper layer. Our setup satisfies dewetting of PDLA layer due to the ~2 times lower intrinsic viscosity of PDLA (0.15 – 0.30 dl/g) than that of PLGA (0.45 – 0.60 dl/g). In addition, dewetting of PDLA films on glass slides confined in PLGA films can be estimated by calculation of Hamaker constants. In this system, $A_{MPS}$ is estimated to $1.8 \times 10^{-22} \text{J}$ (calculated using values in Table 3 and 7 with equation 17) indicated that PDLA films can dewet. In the case of PLGA films on PDLA films immersed in cell culture medium, the protein adsorbed PLGA films are not dewetted and stable since $A_{MPS}$ is estimated to $-7.02 \times 10^{-23} \text{J}$ (calculated using values in Table 3 and 7 with equation 17). The detachment interface can be estimated by the work of adhesion ($W$). In the case of two materials (1 and 2) immersed in a liquid (3), the work of adhesion between 1 and 2 is expressed as:

$$W_{132} = \gamma_{23} + \gamma_{13} - \gamma_{12}$$ (18)

The detachment can occur at the interface of a PDLA-glass surface since the work of adhesion (estimated using values in Table 4.3 and 4.7 and equation (18) of PLGA-
PDLA in cell culture medium (14.3 mJ/m$^2$) is significantly greater than that of PDLA-glass surfaces in cell culture medium (1.4 mJ/m$^2$). For this reason, after tube formations, residues of polymers were not observed on the substrates. It is important to note that this phenomenon can be utilized for the enhancing or retarding of rolling kinetics since dewetting kinetics and the work of adhesion are largely influenced by the surface properties of both polymers, substrates, and their surroundings (i.e., culture medium).

Table 4.7 Contact angles of probe liquids on PDLA and its surface components.

<table>
<thead>
<tr>
<th>Contact angle (°)</th>
<th>DI-water</th>
<th>Methylene iodide</th>
<th>Ethylene glycol</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDLA</td>
<td>66.5 ± 1.1</td>
<td>37.3 ± 0.4</td>
<td>26.2 ± 0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Materials/Liquids</th>
<th>Surface tension and its components (mJ/m$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\gamma$</td>
</tr>
<tr>
<td>PDLA</td>
<td>47.1</td>
</tr>
</tbody>
</table>
4.4.3 Initial Strain Effect on Tube Diameters and Estimation of Initial Strain during Film Generation Process

We believed that the self-rolling of bilayer films was due to the stress relaxation of PLGA layers that was obtained during the film generation process. We speculated that the strain could arise from three steps of the film generation process: compressing of polymer solutions, releasing one of the PDMS sheets, and transferring the films on to passive layers. In fact, all these steps led PLGA films to wrinkle or stretch. As reported, ultraviolet/ozon (UVO)\textsuperscript{191} or oxygen plasma treated\textsuperscript{192} PDMS can obtain wrinkle formation after recovering from the stretched PDMS. However, the wrinkle formation does not have enough energetic actuations to fold bilayer films if the substrate is rigid.\textsuperscript{182,183}

Since it is not realistic to measure strains obtained at each of the steps, we instead investigated the initial strain effect on diameter changes at the same incubation time (~72 h) to confirm that the initial strain on the PLGA films was the cause of the rolling. To examine the initial strain effect on diameter changes, we gave three different bending strains on the PLGA films using three different outer diameters (OD) of circular objects. PLGA films were imprinted in between flat PDMS sheets and one of the PDMS sheets was released. The film attached to the remaining PDMS sheet was bent using several diameters of circular shapes: 20 mL vial (OD = 2.5 cm), 9 cm petri-dish (OD = 9 cm), and 14 cm petri-dish (OD = 14.2 cm), which resulted in ~5, ~3, and ~2 % strains ($\varepsilon_x$), respectively. The PLGA films generated from this method were seen to be intact by observing with a microscope, and it was a safe range since elongation at break of PLGA
was much higher (~31 %)\textsuperscript{193} than our strain range. It is expected that larger strains result in tubes with smaller diameters if the initial strains on the PLGA films are the main cause of rolling. We assumed that the deformations along y- and z-direction were negligible, and non-bent films were set as $\varepsilon_x = 0 \%$. Interestingly, all films that were bent rolled up after 12 h incubation in 37 °C culture medium, while non-bent films kept rolling (Figure 4.27). After 12 h, ~2, ~3, and ~5 % strains resulted in diameters of 73.8 ± 11.4 µm, 50.5 ± 7.5 µm, and 45.7 ± 6.8 µm, respectively (Figure 4.27a and c). We continued the experiment to track the diameter changes for 96 h. Diameters of the tubes were continuously reduced during the incubation time and it reached equilibrium state at 72 h incubation (the diameters were not changed at 96 h incubation). As expected, our data showed that tube diameters were inversely proportional to initial strains at the same incubation times (Figure 4.28).

Our data confirmed that self-rolling of the bilayer films is due to the initial strain applied during the processing of PLGA films. This is also in agreement with an earlier study by Cendula et al.\textsuperscript{188} They revealed that sufficiently large strain differences ($\Delta \varepsilon$) caused rolling of the partially free-hanging bilayer films (Active layer: In\textsubscript{0.1}Ga\textsubscript{0.9}As and Passive layer: GaAs) on a sacrificial layer prepared by selective etching of the layer. According to them, the equilibrium tube radius ($R_{eq}$) can be estimated by the following expressions.
\[ R_{eq} = \frac{\rho d_2}{6\eta \Delta \varepsilon \delta} \]  
(19)
\[ \delta = \frac{d_1}{d_2} \]  
(20)
\[ \rho = (1 + \delta)^3 \]  
(21)
\[ \eta = 1 + \nu \]  
(22)
\[ \Delta \varepsilon = \varepsilon_2 - \varepsilon_1 \]  
(23)

where subscriptions of 1 and 2 are the bottom and upper layer, respectively, \(d\) is the thickness of a film, \(\varepsilon\) is the biaxial strain, and \(\nu\) is the Poisson ratio (\(\nu = (\nu_1 d_1 + \nu_2 d_2)/(d_1 + d_2)\))\(^{226}\). It is important to note that in equations (19) – (23), the radius of a tube is inversely proportional to the strain differences but is proportional to the thickness of the films. These expressions indicate that larger initial strain on the PLGA films result in tubes with smaller diameters as we experimentally verified (figure 4.28). They also indicate that thinner PLGA films resulted in tubes with smaller diameters and vice versa, and appear to agree with our results (Figure 4.29). In their case, the free hanging bilayer films roll towards the bonded area and the rolling is finished when it reaches the interface of bonded and free hanging films. As a result, single tube with several time of rolling of the bilayer films while bilayer films in our approach roll from both edges and finished when the tubes from each sides are contact. Therefore, equation 19 can be modified by incorporating tube width \((w)\) to \(w \approx 4R_{eq} = \frac{\rho d_2}{6\eta \Delta \varepsilon \delta}\), if a thickness of bilayer film is negligible. It was possible to estimate the initial strain on bilayer films using values from Figure 4.28 and equation (19) – (23). For example, \(\Delta \varepsilon\) of bilayer films \((\varepsilon_x = 0 \%\) on PLGA\) consisted of 600 nm PLGA and 330 nm PDLA is estimated to \(~3.7\%\) (using values of \(R_{eq} \approx 13.9 \mu m, \nu_1 = 0.33^{194}\), and \(\nu_2=0.35^{195}\)). Using the same approach,
the actual strains on films with $\varepsilon_x = 2$, 3, and 5 % are estimated to 4.3, 5.0, and 5.3 %, respectively.

Figure 4.27 The dependence of tube diameter on bending strain. The thicknesses of bilayer films were 600 nm and 330 nm, respectively for PLGA and PDLS films. a) After 12 h, films with a $\varepsilon_x = 0$ % were still rolling, while tube formation had been finished for all films with a $\varepsilon_x$ of 2 to 5 %. b) After 48 h, the films with a $\varepsilon_x = 0$ % finally formed tubes, and the tubes resulted from films with a $\varepsilon_x$ of 2 to 5 % reduced in their diameters. c) After 72 h, the diameters of all the tubes slightly reduced. Scale bar: 100 $\mu$m.
Figure 4.28 The dependence of the tube diameter on bending strain at different time. The thicknesses of bilayer films were 600 nm and 330 nm, respectively for PLGA and PDLS films. Values represent mean and standard deviation (n=20).
Figure 4.29 The dependence of tube width on the thicknesses of bilayer films. Values represent mean and standard deviation ($n=20$).
4.4.4 Capability of Cell Encapsulation in Tubes

The feasibility of cell encapsulation in tubes as cell carriers was examined using rat fibroblast (seeding density: $5 \times 10^4$ cells/mL) stained with CellTracker™ Green CMFDA. After 24 h of incubation, ~60% of films became tubes and all cells were still well encapsulated (Figure 4.30a). After 48 h of incubation, all films became tubes and cells were found mostly inside of tubes (Figure 4.30b). We continued incubation of the tubes to verify the stability of tubes and viability of encapsulated cells for 7 days. As shown in Figure 4.30e, diameters of tubes were reduced from $88.3 \pm 12.6 \mu m$ to $53.8 \pm 7.7 \mu m$ during 3 days and increased to $64.3 \pm 10.8 \mu m$ on the 7th day. The decreasing trend of diameters for 3 days could be due to the continuous relaxation of the initial strain on PLGA layers. Later, the reason for the increased diameter (on the 7th day) is not clear at this point. We speculated that water could swell the polymers during incubation and hydrolysis of ester bonds in PLGA and PDLA could cause the slight stretch on the films to be in a stable state. Considering that PLGA has a faster degradation rate than that of PDLA, the recovering strain on the PDLA (by weakening the strains on PLGA films) could exert the effect that caused slightly stretched the tubes. In addition, spreading and proliferation of cells could also stretch the films.
Figure 4.30 Encapsulation feasibility in tubes using rat mouse fibroblasts at (a) 1-day, (b) 2-day, (c) 3-day, and (d) 7-day. Right images are bright field images and left images are fluorescent images after staining with CellTracker™ Green CMFDA. (e) Tube width changes during incubation time for 7 days. Statistical analysis by ANOVA ($n = 20$; *$p < 0.05$). Scale bar: 100 µm.
Figure 4.31 demonstrates viability of encapsulated cells compared with dissociated cells on agarose-coated surfaces (as negative control). At 24 h incubation, viability of encapsulated cells was 90.2 ± 4.2 % and it slightly decreased to 88.7 ± 2.8 % at 72 h and 87.1 ± 5.7 % at 168 h. Compared to encapsulated cells, the viabilities were about 1.4 % lower than that of dissociated cells on agarose, but ~4 % and ~17 % higher for 72 h to 168 h incubation. The results are in a good agreement with viabilities of neural cells encapsulated in hydrogels based self-folding bilayers by others. These results showed that bilayer films provide appropriate surfaces for cell attachment/survival and did not limit diffusion of nutrients for cells.
Figure 4.31 Viability of MEFs encapsulated in tubes for 7 days. Cells encapsulated in tube shows high viabilities during incubation time for 7 days compared to viabilities of cells seeded on agarose-coated surfaces. Significantly different groups are indicated by letter using two-way ANOVA, where “A” represents the highest mean (n = 20, p < 0.05).
4.4.5 Aspect Ratio Effect on Tube Shapes

Since we were interested in several types of tubes that could be injectable, the effect of the aspect ratio of patterns on tube shapes were studied in order to test injectability using them. Previously, we showed that the aspect ratio of 8:1 (W: 500 µm, L: 4 mm) resulted in high aspect ratio of tubes via long-side rolling. We further investigated tube formation with aspect ratio of 2:1 and 4.5:1 of rectangular patterns (W: 500 µm) to examine tube shapes. Since final construct should encapsulate cells, and cells might have an effect on the types of tube formation, mouse embryonic fibroblasts (density: 5 × 10⁴ cells/mL) were seeded on the films. In the case of 2:1 aspect ratio, all side rolling was initially actuated and changed to diagonal rolling. At 24 h, tubes were formed (Figure 4.32a1) and rolling progress was finished (Figure 4.32a2 and a3). Diagonal rolling from all edges first actuated the film with the aspect ratio of 4.5:1 and then long-side rolling actuated. After 24 h, diagonal rolling dominated the films (Figure 4.32b1). After 48h, long side rolled tubes (Figure 4.32b2) and v-shape (Figure 4.32b3) tubes due to the diagonal rolling from two different sides were observed. Our results were in good agreement with and earlier study carried by Stoychev et al.¹¹⁴ They examined folding of rectangular hydrogel based bilayer films with different aspect ratios. They found that high aspect ratios were dominated by long-side rolling when the width and length exceeded the final tube curvatures, and all side rolling started when actuation strain was high. At moderate actuation, diagonal rolling dominated.
Figure 4.32 The rolling dependency of tube on aspect ratio of bilayer patterns with fixed width (500 µm) after seeding MEFs. (a) Diagonal rolling dominates on aspect ratio of 2:1. (a1) after 24 h, edges are still rolling. (a2) and (a3) After 48 h, tubes are done and the edges are perfectly rolled up. (b) Long-side and diagonal-rolling dominates on aspect ratio of 4.5:1. (b1) After 24 h, diagonal rolling to long side rolling are shown. Some films shows diagonal rolling from two different ends. (b2) After 48 h, tubes with long side rolling. (b3) tubes with diagonal rolling from two ends (arrows). (c) Two designs used for injectability. Scale bars: 100 µm for (a3) and others for 400 µm.
4.4.6 Injection Feasibility of Tube

Delivery of the cell in damaged tissue is one of the important issues. It should be noted that transplantation by surgery would not be easy, so injection would be the better choice due to the small size of tubes. Therefore, we examined the injection feasibility of tubes based on that speculation. We evaluated injectability of the tubes using two different types of injection designs: conventional injection (Design A) and injection with gradually decreased diameters to induce laminar flow during the injection (Design B) with a needle gage G 20 (inner diameter: ~600 µm) (Figure 4.12a and b). Figure 4.3 summarizes injectability of the tubes with MEFs made from non-patterned bilayer films. Using Design A, the injectabilities were 67.2 ± 10.4 %, 51.3 ± 8.8%, and 12.2 ± 5.3 %, respectively for aspect ratios of 2:1, 4.5:1, and 8:1. It was found that the injectability was insufficient and needed to be increased for effective delivery. We believed that the low injectabilities were caused not only by the structure of the tubes, but also by the complicated flow pattern caused from different diameters of injection. We speculated that the injectability could be improved by allowing laminar flow during injection. To achieve it, the end of 1 mL of pipette tip was cut (~1cm) and a G 20 needle gage was attached. By using Design B, the injectabilities were improved to 84 ± 7.2 %, 75 ± 6.5 %, and 21 ± 10.3 %, respectively for aspect ratios of 2:1, 4.5:1, and 8:1. Compared to conventional injection, the marked increase of injectability using our design was due to there being no gaps between a needle entrance and the end of the tip, which allowed simpler flow pattern of the fluid.
Figure 4.33 The dependency of injectabilities on design of injection and aspect ratio. Values represent mean and standard deviation ($n = 30$).
4.4.7 Inner Patterned Structures in Tube

To develop more complex inner structures of tubes for directional cell growth, we investigated tube formation using imprinted PLGA films with parallel patterns (20 µm of spacing and 2 µm of height) as an active layer. Three different types of rectangular bilayer films were prepared in this study: perpendicular, parallel, and diagonal to the long side of the films (Figure 34a1, b1, and c1). For perpendicular to long side of the bilayer films, long side rolling dominated similar to flat bilayer films with aspect ratio of 8:1 (see Figure 4.25). The direction of PLGA strips inside of tubes (formed at 24 h) was perpendicular to the tubes (Figure 4.34a2). At 48 h incubation, the tubes displayed a slight contraction (Figure 4.34a3). Next we investigated tube formation using strips parallel to the long side of the bilayer films. Interestingly, it underwent short-side rolling (Figure 4.34b2), which was totally different phenomenon found on the high aspect ratio of bilayer films. After 24 h, the short-side rolling of bilayer films resulted in ribbon shaped tubes (Figure 4.34a3). It is known that short side rolling is rarely observed since the force of later long-side rolling strong enough to dominate the short-side rolling due to the aspect ratios of the rectangular films.\textsuperscript{117,197} Considering the timing of tube formation of non-patterned (~48 h) and patterned bilayer films (~24 h), our unique inner patterned structures enhance the ability of the short side rolling to overcome long side rolling dominance. Higher bending strain helps to overcome adhesion between PDLA and substrate, therefore, it inhibits switch over to long-side rolling.
Figure 4.34 The dependency of rolling direction on pattern direction (a1) perpendicular, (b1) parallel, and (c1) diagonal to long side of bilayer films with MEFs seeding. (a) diagonal direction of patterns results in long side rolling. The tub tube formation at (a2) 24 h and (a3) 48 h. (b) Parallel direction of patterns results in short side rolling. (b2) short side rolling at 12 h, and (b3) ribbon shape of final structure of bilayer films at 24h. (c1) Diagonal direction of patterns at initial state. Rolling starts from diagonal rolling for all aspect ratio of bilayer films: (c2) 4:1 and (c3) 8:1 at 4 h. (c4) After 12 h, long sides dominates on high aspect ratio and cells grow along with the direction of the inner patterns. (c5) 4:1 aspect ratio results in diagonal rolling (arrows) and (c6) results in long side rolling at 24 h incubation.
Finally, the tube formation via a diagonal direction of PLGA strips was investigated. In this work, we utilized bilayer films with different aspect ratios of 2:1, 4:1, and 8:1. At 4 h incubation after seeding cells, diagonal rolling started from the edges for all aspect ratios (Figure 4.34c2). For aspect ratios of 2:1 and 4:1, rolling direction was not switched and tubes via diagonal rolling resulted at 24 h incubation (arrows in Figure 4.34c5). For films with aspect ratio 8:1, it switched to long-side rolling at 4 h (Figure 4.54c3). At 12 h incubation, rolling was progressed and cells were well attached and aligned with the patterns (Figure 4.34c4). At 24 h, tube formation was finished, resulting in helical structures of inner strips (Figure 4.34c6). The helical structures are beneficial since it resembles structures of native blood vessels. The fabrication of injectable pre-formed blood vessels will be introduced in the following section.

4.4.8 Co-culture of SMCs and ECs in Bilayer Films

We showed that 2-D bilayer films became several types of 3-D tubes and verified it could be utilized for injectable cell carriers. Since the tubes have high aspect ratios, which are similar to native blood vessels, we focused on encapsulation of ECs and SMCs in this section. Later we will show its potential to generate pre-formed blood vessels based on the hypothesis that the pre-formed blood vessel structures could enhance the tissue regeneration.

We first utilized non-patterned bilayer films to verify the capability of encapsulation of SMCs and ECs and viability of blood cells in vitro (Figure 4.35). For ECs encapsulated in tubes, the tubes maintained smooth surfaces while SMCs
encapsulated tubes were distorted. We noticed that SMCs were widely spread, therefore, cell behavior could affect the tube structures. However, it did not change the rolling direction and followed the rolling direction depending on aspect ratio of initial patterns as verified earlier. For example, low and high aspect ratio resulted in diagonal rolling and long side rolling, respectively.

We also verified viabilities for 7 days incubation in tube and compared that with those of dissociated cells on agarose-coated surfaces as negative controls. Before seeding cells, the initial viabilities of SMCs and ECs were 95.8 ± 3.5 % and 94.2 ± 2.4 %, respectively. As shown in Figure 4.36 viabilities of SMCs and ECs showed decreasing trends from 92.2 ± 4.8 % (at 24 h) to 71.8 ± 7.1 % (at 168 h) for SMCs and from 91.4 ± 6.2 % (at 24 h) to 65.9 ± 3.9 % (at 168 h) for ECs. Unlike the viabilities of the negative controls, those of encapsulated cells in tubes did not change much for 7 days incubation. After 24 h incubation of the tubes, viability of SMCs was measured at 93.4 ± 2.5 % and decreased to 91.4 ± 6.2 % at 168 h incubation. Similarly, viability of ECs was measured to 92.8 ± 5.5 % at 24 h and decreased to 91.1 ± 7.3 % at 168 h incubation. We have previously shown that tubes did not significantly limit the diffusion of nutrients to MEFs (Figure 4.32), and the same results were confirmed in this study.
Figure 4.35 Encapsulation of (a) SMCs and (b) ECs and viability in tubes for 7 days. Bright field (left) and fluorescent (right) images of SMCs stained with CellTracker™ Green CMFDA for live cells and Hoechst blue for nuclei encapsulated in tubes at (a1) 1 day, (a2) 2 days, and (a3) 3 days as well as those of ECs at (b1) 1 day, (b2) 2 days, and (b3) 3 days.
Figure 4.36 Viabilities of (a) SMCs and (b) ECs on agarose (negative control) and in tubes from non-patterned bilayer films (pattern 500 µm × 4 mm). Significantly different groups are indicated by letter using two-way ANOVA, where “A” represents the highest mean (n = 20, p < 0.001).
We further mimicked the native structure of blood vessels using self-rolling bilayer films. Native blood vessels have a structure of SMC layers covering the ECs layers. The helix composed of the SMCs and fibers are inclined to the centerline of blood vessels (Figure 4.37). For large blood vessels (e.g., arteries), the angle of the inclined helix winding to the longitudinal axis of the blood vessel is $30^\circ - 50^\circ$. The angles are increased as the diameters of blood vessels decrease (e.g., $\sim 90^\circ$: small arterioles). To incorporate these native structures in tubes via self-rolling bilayer films, we decided to culture SMCs on diagonally patterned bilayer films until it achieved the full coverage of SMCs layers on the films, then seeded ECs on them (Figure 4.38). Before achieving tube formation (with seeding SMCs and ECs), we evaluated SMCs alignment on the patterned PLGA films. After rolling of the films, it might not be possible to evaluate the alignment of SMCs. Therefore, single PLGA films were directly generated on substrates by imprinting with 10 wt % PLGA solution in acetone with the featured PDMS stamp used in this study. As shown in Figure 4.40, the bright field images (Figure 4.39a) did not show clear cell spreading to verify cell alignment, which might be due to the patterns. However, fluorescent images (Figure 4.39b) showed clear cell spreading after 24 h incubation. To verify cell alignment, we measured the SMCs alignment ($\theta < 15^\circ$) and found it to be $89.8 \pm 8 \%$. Therefore, it was verified that the tracks induced the cell alignment while SMCs on smooth PLGA films grew randomly. In addition, we noticed that ECs stained with Hoechst blue were well attached on SMCs layers (Figure 4.39c).
Figure 4.37 Inclination of SMCs depending on types of blood vessels: (a) large size blood vessels and (b) small size blood vessels.

Figure 4.38 Schematic of co-culture of ECs and SMCs.
Figure 4.39 SMCs alignment on patterned PLGA films. (a) SMCs on smooth PLGA films (b) Bright field image of SMCs alignment on parallel striped patterned PLGA films (spacing ~20 µm) on glass slides. (c) SMCs stained with CellTracker™ Green CMFDA are growth through the direction of the strips and (d) ECs stained with Hoechst blue are well attached on SMCs. SMCs (density: $1 \times 10^5$ cells/mL) were incubated for 20 h, and then ECs ($1 \times 10^5$ cell/mL) were seeded on SMCs layer. Images were taken after 4 h incubation. Scale bar: 100 µm.
Finally, we attempted to fabricate pre-formed blood vessel generation by co-culture of SMCs and ECs on diagonal patterned bilayer films based on the experimental design (see Figure 4.38). As shown in Figure 4.40a, cells were attached on the bilayer films and cell detachment during rolling was not observed after 4 h incubation. At this time, we decided to seed ECs to achieve co-culture of two types of cells inside of tubes unless the SMCs had not spread yet. After 24 h incubation, tube formation was done with inner helical structures of strips and both cells were well encapsulated (Figure 4.40b and 4.40c). After 48 h (Figure 4.40d, 4.40e, and 4.40f), tube formation was maintained.

As noticed earlier, spreading of SMCs encapsulated in non-patterned bilayer films could distort the tube shapes, however, it was reduced by utilizing inner patterned bilayer films. We speculated that the pattern direction allowed directional growth of cells and diminished the distortion. Unless we successfully co-cultured two types of cells within the tubes, several concerns still exist. First, tubes that rolled from two ends of edges resulted in two inner channels in the tube (see Figure 4.24). It is a different structure than that of the native blood vessels that only have one channel. Second, tubes are formed by rolling several times, therefore, the final structures of the tubes consist of with several layers of two types of cells. In native tissues, there is only the innermost layer of ECs, covered with SMCs. Lastly, SMCs are not fully spread on PLGA surfaces before seeding ECs. The problems that arise here could be solved by further studies. For example, possibly changing the thicknesses of two layers and making it narrower, might achieve rolling only once. In this case, the maximum number of rolling \( N_{\text{max}} \) of tubes from each side can be estimated by following the expression we developed (details are summarized in APENDIX II),
where, \( L \) is the initial width of bilayer films, \( R_{eq} \) is equilibrium radius of tubes, \( n \) is number of rolling, \( h \) is a total thickness of bilayer films. Equations (19) – (24) can be utilized for determining width, thickness, and strain differences of bilayer films to achieve tubes with specific diameters and number of rolling of the bilayer films. For timing of tubing formation, not only controlling the thicknesses of two layers, but also altering surfaces properties could delay the tubing. In this case, altering the surface properties of substrate, polymers, and cell culture medium could be delayed until SMCs are spread. In addition, SMCs are seeded after encapsulation of ECs in tubes. After degradation of biodegradable polymer layers, SMCs and ECs will contact each other.
Figure 4.40 Pre-formed blood vessel generation. (a) 4 h after seeding SMCs on diagonal patterned bilayer films. ECs are seeded at this time. (b) After 24 h, tube formation is finished resulting in helical inner patterned tubes with (c) encapsulation of two types of cells: green fluorescent – SMCs and blue fluorescent – ECs. (d) After 48 h, tube formation maintains tube formation and (e) two cells are well encapsulated inside of the tubes. (f) Confocal image of 3 days incubated tubes with encapsulated two types of cells. Scale bar: 500 µm for (a) – (c) and 100 µm for (d) – (f).
4.4.9 Summary

In conclusion, we demonstrated several types of tube formation via self-rolling of fully biodegradable bilayer films that can be achieved by a simple imprinting method. The mechanism of self-rolling bilayer films was programmed strains equipped on PLGA films (active layer) as well as the strain gradient on the PLGA films produced due to the PDLA layer. The strain differences was estimated to ~3.7 % during the fabrication of PLGA films (600 nm) on PDLA films (330 nm). $N_{\text{max}}$ on each side was ~2.7 times in this case. In addition, the PDLA layer plays a role in forming a temporal adhesive to induce controlled detachment from the substrates. Since the detachment was first caused at the four edges of the films, rolling direction was switched to long- or diagonal-rolling depending on initial aspect ratios of the bilayer patterns. In addition, by utilizing a featured PDMS pad, complex patterned structures (perpendicular, diagonal, and parallel, to long side of rectangular films) inside of tubes were simply achieved. We confirmed that it could be utilized for an injectable cell carrier with respect to 7 days viability (~85 %) and injectability (~80 % using 2 mm length tubes) using G 20 needle with our unique design of syringe. We further created pre-formed blood vessels by co-culture of SMCs and ECs on diagonal inner patterns on bilayer films. However, rolling started (at 4 h) without achieving monolayer of SMCs on the films. By seeding ECs at this time, the final cells encapsulated without two separate layers (ECs on SMCs). In addition, tubes are formed with several times of rolling resulting in alternating layers SMCs and ECs. For delayed rolling, more studies should be needed with respect to slower dewetting of PDLA layers by changing surface properties of substrates and cell culture medium.
5.1 Concluding Remarks

This work presents two different cell delivery constructs that mimic natural tissues. Current delivery systems are reviewed with respect to their structures and processing techniques, including advantages and disadvantages. While current systems do not truly incorporate fibrous or high aspect ratios of structures due to their processing limitations or complications, the techniques in this study are developed to incorporate such structures. Compared to conventional techniques, the techniques developed in this study are not only different approaches, but also simpler and more cost-effective.

The first study was focused on understanding dewetting behaviors of thin PLGA films under aqueous solutions, which are fundamental for fabricating the two different constructs. This study was different from conventional studies, because it was examined by heating above $T_g$ of polymers at ambient conditions. At ambient conditions, destabilizing forces are primarily van der Waals interactions. However, in aqueous solutions, two interactions are competing as a result of the properties of the aqueous solutions. In DI-water and PBS, dewetting of PLGA films thinner than 30 nm were mainly controlled by electrostatic interactions, while van der Waals attractions were dominant destabilizing forces for thicker films ($> 30$ nm). In the 0.4 wt % BSA solution
and culture medium, electrostatic interactions were destabilizing forces and van der Waals interactions were stabilizing forces.

The first injectable micro-construct developed in this study was a fibrous cell/polymer bundle. The patterning method in this study was a masterless method, which is easier, more convenient, and cost-effective compared to current pattern generation by soft lithography or photolithography. Parallel striped patterns on PLGA films became fibrous films due to the rupture of the thin layer in-between thick ridges by dewetting. To induce more cells and assure the generation of cell/polymer constructs, agarose-coated surfaces were utilized. The main differences between these constructs and conventional cell carriers are cell distributions and structures of the constructs. Cells are grown in and outside of the constructs, which have not been achieved by current methods. The constructs have structures similar to that found in natural tissue, because it has fibrous and high aspect ratio of the structures. Furthermore, cells interconnected in and outside of the constructs will allow direct cell-to-host organ contact. Since it has structural integrity compared to dissociated cells, it showed a protective effect by maintaining initial viability after injection through a G 20 needle. The in vitro and in vivo retention studies proved the constructs have a better retention compared to the dissociated cells due to its structural integrity. However, one of the disadvantages is its low injectability using a conventional injection, since that produces complicated flow patterns. To overcome this challenge, we developed a new design of injection which showed reasonable injectability, however, it does need to be proofed for clinical use.
The second construct developed in this study was a 3-D tubular construct via self-rolling bilayer films. The formation was achieved by programmed strains, which actuate when adhesive layers lose the adhesion from substrates. Complex inner structures can be achieved by incorporating parallel patterns imprinted by a featured stamp. Several types of tubes can easily create different size (i.e., aspect ratio) bilayer patterns. Several types of cells were able to be encapsulated and viabilities of the encapsulated cells for 7 days were not largely decreased. Compared to hydrogel based current approaches, which bend by volume expansion due to up-taking large amount of water, our approach offers a thinner wall of tubes. In addition, as hydrogels thicken, the total thickness of the tube is also increased and that could have a negative effect on the diffusion of nutrients for cells. We further attempted to generate this construct approach for a potential candidate for injectable pre-formed blood vessels by co-culture of rat SMCs and ECs. One of the problems was controlled rolling kinetics. To generate a ECs layer on a SMCs layer, rolling of the bilayer films should not be actuated. However, the current stage of our design does not offer enough delayed time of rolling. Therefore, the remaining challenge in this technique is to elongate the stable time of bilayer films under culture medium condition.

In summary, two different types of high aspect ratio of injectable micro-constructs were generated. The advantage of these micro-constructs is that they truly mimic native tissues and its fabrication technique can be utilized by simple and cost-effective methods. Due to the fact that these carriers are totally different structures, these noble designs could open a new paradigm to generate cell delivery vehicles.
5.2 Future Studies

Future studies include the following:

1. This work mainly utilized PLGA and PDLA for the fast degradation \textit{in vivo}. However, other types of polymers have not been examined to achieve our proposed constructs. To provide long-term structural stability \textit{in vivo}, other polymers (e.g. polycarprolactone) should be tested.

2. This work mainly focused on the fabrication of injectable cell polymer constructs using conventionally available biodegradable polymers. However, the effect of surface modifications on cell-polymer interactions has not been studied. In the future, surface modification with RGD-peptide or silanization should be studied as well as characterization of cell-polymer interactions.

3. Several types of self-rolling bilayer films were generated in this study. However, kinetics for controlled rolling were not examined. In the future, kinetics for tube formation should be studied, including the surface modification of substrates (e.g. PEG-silane), use of different molecular weight of adhesive layer, and changing surface properties of liquid environment by adding bio-surfactant.

4. Two types of scaffolds have high aspect ratio and may be utilized to generate injectable pre-formed tissues that the most of current injection based approaches have not examined. In this case, \textit{in vivo} study can be carried out based on the hypothesis that pre-formed structures can enhance the tissue regeneration.
5. Tubes via self-rolling bilayer films can be utilized for controlled release of cells or drugs. Since the formation of tubes are possible due to the strain gradient caused by passive and active layers, removal of the passive layer will flatten the active layer as a result of the faster degradation rate of the passive layer as compared to the active layer. For material setup, bilayer films can be composed of poly(lactic-co-glycolic acid) for an active layer and poly(glycolic acid) for a passive layer.
REFERENCES


Pan, Z.; Ding, J. Poly(lactide-co-glycolide) porous scaffolds for tissue engineering and regenerative medicine. *Interface Focus* 2012, 2, 366–377.


APPENDICES
APPENDIX A

DETERMINATION OF SURFACE TENSION AND ITS COMPONENTS OF AQUEOUS SOLUTIONS USING LIQUID-LIQUID INTERFACIAL TENSIONS

The purpose of this study is to provide a method that can determine surface tension and its components of aqueous solutions. We hypothesized that lower interfacial tensions between the aqueous solution and probe liquids would allow us to obtain more accurate surface tensions. One reason is that the lower interfacial tension would likely provide a smaller driving force for the solutes to transport to or from the water/oil interface. To verify our hypothesis, the surface tensions of several aqueous solutions: water, 3.5 wt % salt, 30 wt % sucrose, phosphate buffered saline, and a cell culture medium were determined. Probe liquids of benzaldehyde(BA), ethyl acetate(EA), ethyl ether(EE), benzene(BZ), and octane(OC) were selected based on their interfacial tensions with water and their polarity. In general, three probe liquids are needed to solve for the Lifshitz – van der Waals and acid/base components of the surface tension. Four combinations of the probe liquids were compared: BA-EA-EE, BZ-EA-EE, OC-EA-EE, and OC-BZ-EE, based on a sensitivity analysis with a 5 % of error to the interfacial tensions of water and each of the probe liquids. Experimentally, the pendant drop method was utilized to determine the interfacial tensions. For water, due to the absence of solutes, all four combinations lead to similar values of surface tension and its components. However, when the solutes are presented, the values varied with the
combination of the probe liquids used. More accurate values were indeed found to be resulted from the combination (i.e. BA-EA-EE) that had lower interfacial tension with water.

A.1 Introduction

Aqueous media are essential for the survival of living matters and proper functions of biological systems. To understand their fundamental roles, the properties of the aqueous solutions have to be first determined and then utilized. One of the critical properties of a liquid is surface tension.

The surface tension determination of a liquid mixture is not trivial. While some experimental methods, such as capillary rise, pendant drop, and drop weight, have been developed for measuring surface tension of pure liquids with a great accuracy, these methods might not be able to obtain the reasonable surface tension of a liquid mixture. How molecules of different liquids distributed at the surface and within the bulk of the liquid mixture can greatly affect the measured surface tension value. Such distribution should be examined while applying a method to determine the surface tension or interfacial tension of a liquid mixture. Also, in most cases, a simple total surface tension value is not as useful as knowing the different components of the surface tension, since the components are the ones used to determine the interactions between the liquid and other substances.

Since most substances have a polar and an apolar property, van Oss and co-workers defined surface tension ($\gamma^\text{tot}$) as the sum of polar component ($\gamma^\text{AB}$), due to acid-
base (AB) interactions, and apolar component ($\gamma^\text{LW}$), due to the Lifshitz-van-der Waals (LW) interactions.\textsuperscript{122–124,203} They have developed several approaches, both experimentally and theoretically, to obtain the surface tension components. For a pure liquid, contact angles of the liquid on a pure apolar solid surface could be used to obtain its $\gamma^\text{LW}$ and its $\gamma^\text{AB}$ could then be estimated by subtracting $\gamma^\text{LW}$ from $\gamma^\text{tot}$ of the liquid, which could be measured with common methods such as capillary rise or pendant drop. They also introduced a different method by encasing a liquid in gels with different gel concentrations.\textsuperscript{203} The contact angles of three known test liquids on these gels were measured and the surface tension components were then calculated from the contact angle values, and finally the surface tension component values were extrapolated to zero gel concentration to obtain $\gamma^\text{LW}$ and $\gamma^\text{AB}$ of the liquid encased.

However, the methods based on contact angle measurements cannot be easily applied to determine surface components of liquid mixtures, especially aqueous mixtures, due to the attraction/repulsion of solutes at the liquid-air interface and in some cases the adsorption of solutes to the solid surface.\textsuperscript{204} The interactions/adsorption would complicate the correct measurements of contact angles; also contact angles of a liquid mixture were not recommended and could be invalid for the determination of surface tension and its components of a liquid mixture.\textsuperscript{205} This invalidity has been noticed previously. For example, the surface tension of a solid determined with pure liquids and that determined by aqueous mixtures were different.\textsuperscript{206,209} In fact, according to van Oss, it is fundamentally wrong to apply the contact angles of a liquid mixture in Young’s equation for surface energy/tension evaluation.\textsuperscript{197} However, many researchers have
commonly employed the approach,\textsuperscript{206–211} due to, probably, the simplicity of the measurement or unavailability of proper alternative methods.

To determine $\gamma^{\text{LW}}$ and $\gamma^{\text{AB}}$ of an aqueous mixture, we believe a key is to reduce the effect of repulsion or attraction of the substance in the aqueous mixture from/to the interface (see Figure A.1) as the surface/interfacial tensions are being measured. Since air is one of the most hydrophobic substances (liquids form 180 degree of contact angle with air),\textsuperscript{212–216} to reduce hydrophobic attractions of the hydrophobic or amphiphilic substances dissolved in water towards air, liquid-liquid interfacial tensions instead of liquid-air interfacial tension (i.e. liquid surface tension) could be better choices. Also, by using a solvent that forms a lower interfacial tension with water, the migration of substance to the interface due to hydrophobic attractions could be lessened. Similarly, in the case that a bulk contains a high-energy substance that would tend to move away from the liquid/air interface, an under-estimated surface tension could result. When a liquid that forms a lower interfacial tension with the aqueous phase is used, the tendency of the substance moving away from the interface could be reduced, allowing a more accurate measurement of the interfacial tension.

In this paper, we hypothesized that the surface tension and its components of an aqueous solution could be more accurately determined by using interfacial tensions of the aqueous solution and organic solvents (i.e. probe liquids) that result in smaller interfacial tensions. With lower interfacial tensions, the tendency of molecules in the bulk solution to transport from/to the interface (to lower the interfacial tension) could be less severe,
thus allowing the interfacial tension to be measured more accurately, leading to more precise estimation of the surface tension and its components of the aqueous solution. The pendant drop method was employed to obtain the interfacial tensions. Our results indeed showed that by using probe liquids that had lower interfacial tensions with water, more accurate values of surface tension and its components of several aqueous mixtures were obtained.

Figure A.1 Two scenarios could occur at the water-air interface for an aqueous solution containing solutes: (a) A depletion zone due to the repulsion of hydrophilic solute molecules by air, (b) a concentrated zone due to the hydrophobic attraction of solutes to air.
A.1.1 Background

As mentioned earlier, van Oss and co-workers have defined that the surface energy or tension ($\gamma^\text{tot}$) of a solid surface or a liquid as $\gamma^\text{tot} = \gamma^\text{LW} + \gamma^\text{AB}$. $\gamma^\text{AB}$ can be expressed as: $\gamma^\text{AB} = 2\sqrt{\gamma^+\gamma^-}$, where, $\gamma^+$ and $\gamma^-$ stand for the electron acceptor and electron donor parameter, respectively.\textsuperscript{122-124,203} When two different materials (i, j) are interacting with each other, the energy of interactions ($\Delta G_{ij}$) can be written as,

$$\Delta G_{ij} = -2 \left( \sqrt{\gamma_i^\text{LW}\gamma_j^\text{LW}} + \sqrt{\gamma_i^+\gamma_j^-} + \sqrt{\gamma_j^+\gamma_i^-} \right) = \gamma_{ij} - \gamma_i - \gamma_j \quad \text{(A.1)}$$

and the interfacial tension ($\gamma_{ij}$) becomes:

$$\gamma_{ij} = \gamma_i + \gamma_j - 2 \left( \sqrt{\gamma_i^\text{LW}\gamma_j^\text{LW}} + \sqrt{\gamma_i^+\gamma_j^-} + \sqrt{\gamma_j^+\gamma_i^-} \right)$$

$$= \left( \sqrt{\gamma_i^\text{LW}} - \sqrt{\gamma_j^\text{LW}} \right)^2 + \gamma_i^\text{AB} + \gamma_j^\text{AB} - 2 \left( \sqrt{\gamma_i^+\gamma_j^-} + \sqrt{\gamma_j^+\gamma_i^-} \right) \quad \text{(A.2)}$$

When a mono-polar liquid (i) containing only $\gamma_i^-$ is used as the probe liquid, equation (A.2) can be simplified to,

$$\gamma_{ij} = \left( \sqrt{\gamma_i^\text{LW}} - \sqrt{\gamma_j^\text{LW}} \right)^2 + \gamma_j^\text{AB} - 2 \sqrt{\gamma_i^-\gamma_j^+} \quad \text{(A.3)}$$

where, $\gamma_i^\text{LW}$, $\gamma_j^\text{AB}$, and $\gamma_j^+$ are three unknowns. To solve for these three unknowns, interfacial tensions of 3 mono-polar liquids (i.e. i) and the liquid of interest (i.e. j) are necessary to obtain the total surface tension of liquid j.
A.2 Experimental Details

A.2.1 Materials and Equipment

De-ionized (DI) water was purified in house with a conductivity value of 1 – 2 µS. Octane was from Acrose Organics. Benzene and benzaldehyde were from Sigma Aldrich. Ethyl ether and ethyl acetate were from Fisher Scientific and EM Science, respectively. They were all HPLC grade and filtered using 0.45 µm PTFE filters prior to use. Phosphate buffered saline (PBS), sodium chloride, and sucrose were from Sigma-Aldrich and Fisher Scientific, respectively. The cell culture medium was prepared by mixing 85 v % knockout- Dulbecco’s modified Eagle medium (Invitrogen, Carlsbad, CA) and 15 v % fetal bovine serum (Thermo Scientific, Waltham, MA).

The equipment used included a Gilmont syringe containing a 2.0 mL glass barrel (from Cole Parmer) connected to a blunt end gauge 22 stainless steel needle (Popper & Sons, Inc.), a Ramé-Hart contact angle goniometer (model 100-00) with a CCD video camera, and an image capturing device and its software (Diamond VC500, One Touch Video Capture).

A.2.2 Sensitivity analysis on solvent selection

The sensitivity analysis, by incorporating 5 % of error to the potentially measured interfacial tensions between water and each organic solvent (probe liquid), was carried out to determine the range of potential surface tension and its components using equations (A.3). Benzaldehyde (BA), ethyl acetate (EA), ethyl ether (EE), benzene (BZ), and octane (OC) were selected based on their interfacial tensions with water and their
polarity. Four combinations of the probe liquids, BA-EA-EE, BZ-EA-EE, OC-EA-EE, and OC-BZ-EE, were used for the analysis.

A.2.3 Interfacial tension measurement

Experimentally, the pendant drop method, one of the most convenient and precise methods for determining surface or interfacial tension\textsuperscript{200-202} was applied for measuring the interfacial tensions at 25 ± 1 \degree C. DI water, PBS, 30 wt % sucrose solution, 3.5 wt % sodium chloride solution, and cell culture medium were selected as five model aqueous mixtures. To obtain the total surface tension, a pendant drop of each of these solutions, filled in the Gilmont syringe barrel, and was formed at the needle tip by dispensing 100 – 200 \( \mu \)L of the liquid in air. The shape of the drop was captured and the needed dimensions were measured by using Image J. To measure the interfacial tension of an aqueous solution and an organic solvent, the solvent was placed in to a quartz spectrometer cuvette, and an aqueous pendant drop was formed inside the organic solvent (in most cases, the organic solvent had a low density than that of water). To avoid the evaporation of the liquids, the opening of the cell was covered with a Paraffin tape.

The surface or interfacial tension was determined using the expression below,

\[
\gamma_{ij} = \frac{\Delta \rho g D e^2}{H} \tag{A.4.}
\]

where, \( \gamma_{ij} \) refers the interfacial tension of liquids i and j, \( g \) is the gravitational acceleration, \( De \) is the equatorial diameter, and \( H \) is the shape factor that is a function of \( Ds/De \), with \( Ds \) being the diameter measured at a distance \( De \) up from the bottom of the drop. The value of \( H \) can be obtained from empirical relationships.\textsuperscript{121} More than 30
drops of each aqueous solution/organic solvent pair were examined to determine the interfacial tensions.

A.3 Results and Discussion

A.3.1 Sensitivity analysis of liquid-liquid interfacial tensions on surface tension determination.

Five probe liquids were first chosen based on their solubility in water and their interfacial tensions with water. The details of these values, obtained from existing literatures, are summarized in Table A.1. The lowest and highest interfacial tension of the probe liquid with water was 6.8 mJ/m$^2$ for ethyl acetate (EA) and 51.0 mJ/m$^2$ for octane (OC). The other three solvents were chosen, in addition to EA and octane, to span the entire interfacial tension range: $\gamma_{\text{water/ethyl ether (EE)}} = 10.7$ mJ/m$^2$ and $\gamma_{\text{water/benzaldehyde (BA)}} = 15.5$ mJ/m$^2$, and $\gamma_{\text{water/benzene (BZ)}} = 34.9$ mJ/m$^2$.

To assess the applicability of using liquid-liquid interfacial tensions to determine the surface tension and its components of aqueous solutions, the sensitivity analysis was first carried out by incorporating up to a 5% error in the interfacial tension value for each probe liquid and water. Four combinations of probe liquids were selected and their surface tensions and components are also summarized in Table A.1. The combination that had the lowest interfacial tensions with water was BA-EA-EE (15.5, 6.8, and 10.7 mJ/m$^2$, respectively). BZ-EA-EE gave the second lowest interfacial tensions (34.9, 6.8, and 10.7 mJ/m$^2$), and OC-EA-EE resulted in the third lowest interfacial tensions (51.0,
6.8, and 10.7 mJ/m²). The combination had the highest interfacial tensions (51.0, 34.9, and 10.7 mJ/m²) was OC-BZ-EE.

In reality, when surface tension of water would be experimentally determined using the liquid-liquid interfacial tension method, errors could result in all three interfacial tensions with different magnitudes. But, it was difficult to present all three errors vs. the deviations from the literature surface tension of water in one chart. As such, a chart composed of no error in two probe liquids and % error in one probe liquid was used (Figure A.2), we believed the chart would still point out the limitations of each combination and the effect of a probe liquid on the estimation of a surface tension. For a combination consisting of three mono-polar probe liquids (Figure A.2a, b, and c), a more deviation was resulted in $\gamma^{AB}$ than in $\gamma^{LW}$. On the other hand, a combination consisting of two mono-polar liquids and one apolar liquid (i.e. OC) that formed the highest interfacial tension with water, a significant higher error was resulted in $\gamma^{LW}$ than in $\gamma^{AB}$ (Figure A.2d, e, and f).

Table A.1 Probe liquids used for this study with their surface tension information are summarized. Interfacial tensions ($\gamma_{ij}$) of water and each of the probe liquids are calculated based on the reported surface tension values.

<table>
<thead>
<tr>
<th>Probe liquid</th>
<th>Surface tension and its components (mJ/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\gamma^{tot}$</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>23.9</td>
</tr>
<tr>
<td>Ethyl ether</td>
<td>17.0</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>38.5</td>
</tr>
<tr>
<td>Benzene</td>
<td>28.9</td>
</tr>
<tr>
<td>Octone</td>
<td>21.6</td>
</tr>
</tbody>
</table>

Note: “a”: Interfacial tension ($\gamma_{ij}$) of water and each of the probe liquids are calculated based on the reported surface tension values using equation A.3.
Figure A.2 The sensitivity analysis on the surface tension and its components using the interfacial tensions of the combination of BA-EA-EE and OC-BZ-EE, is shown. Deviations (the differences between the calculated values and the reported values of water ($\gamma_{\text{water}}=72.8$, $\gamma_{\text{water,BA}}=21.8$, and $\gamma_{\text{water,EE}}=51.0$) were plotted ($\gamma_{\text{tot}}$ (—), $\gamma_{\text{water}}$ (---), and $\gamma_{\text{AB}}$ (……)) for different errors in each of the interfacial tensions. a) Error in $\gamma_{\text{water,EA}}$. b). Error in $\gamma_{\text{water,BA}}$. c) Error in $\gamma_{\text{water,EE}}$. d) Error in $\gamma_{\text{water,BA}}$. e) Error in $\gamma_{\text{water,BZ}}$. f) Error in $\gamma_{\text{water,OC}}$. 
A.3.2 Measured Surface and Interfacial Tensions

Aqueous solutions selected for this study were DI-water (to serve as reference I), 30 wt % sucrose in water (to serve as reference II, since the values have been reported by others\textsuperscript{206}), 3.5 wt % NaCl in water, PBS, and a cell culture medium. Surface tension of each solution was firstly determined by the pendant drop method in air. Then the interfacial tensions between these aqueous solutions and the five selected probe liquids were measured, also by pendant drop, and used to estimate the surface tension and its components of each of the aqueous solutions. The measured surface tension and the interfacial tensions are reported in Table A.2.

The measured surface tension of water (in air) was 73.0 mJ/m\(^2\), which was in a good agreement with the reported value (72.8 mJ/m\(^2\))\textsuperscript{123,124}. The surface tension of the sucrose solution, PBS, and 3.5 wt % salt solution was 74.4 mJ/m\(^2\), 71.8 mJ/m\(^2\), and 73.5 mJ/m\(^2\), respectively, all of them were very close to the corresponding values (74.2 mJ/m\(^2\),\textsuperscript{206} 69.5 – 70.5 mJ/m\(^2\),\textsuperscript{218,221} and ~ 74 mJ/m\(^2\))\textsuperscript{220–223} reported or calculated. The surface tension of the culture medium determined in air was 64.7 mJ/m\(^2\), which was also expected. This lowered surface tension, as compared to that of pure water, was likely caused by the migration and assembly of proteins to and at the air/water interface. The slight deviations in surface tensions of sucrose and salt solutions from that of water were probably the results of potential affinity or aversion of solute molecules towards the water/air interface.
The differences in surface tension changes as a function of time for the culture medium and the other solutions (water and the sucrose solution) are shown in Figure A.3. For water, the surface tension was ~ 73.0 mJ/m$^2$ over the entire period (20 minutes) of measurement. For the sucrose solution, a slight increase, from 74.4 mJ/m$^2$ to 75.4 mJ/m$^2$, in its surface tension was observed at ~ 20 – 50 seconds after the drop was formed. Then, the value basically stayed at 74.5 mJ/m$^2$ throughout the rest of the experiment. For the cell culture medium, however, a quick reduction in surface tension was observed from 0 (~ 66 mJ/m$^2$) to ~ 200s (~ 51.0 mJ/m$^2$) after the drop was formed, and then the value maintained at ~ 51.0 mJ/m$^2$ for the rest of the duration (up to 20 minutes).

The measured interfacial tensions of aqueous solutions and probe liquids are presented in Table A.2, which shows similar trends with time as the surface tensions. The measured values were 51.3, 35.1, 16.0, 10.7, and 6.8 mJ/m$^2$, respectively for $\gamma_{\text{water/OC}}$, $\gamma_{\text{water/BZ}}$, $\gamma_{\text{water/BA}}$, $\gamma_{\text{water/EE}}$, and $\gamma_{\text{water/EA}}$, which were all in a good agreement with the reported data. The interfacial tensions for most of the aqueous solutions and the probe liquids were slightly deviated from those of water and the probe liquids, while a greater deviation for the values of cell culture medium were again observed. For example, the values of $\gamma_{\text{medium/OC}}$, $\gamma_{\text{medium/BZ}}$, and $\gamma_{\text{medium/BA}}$ are 27.4, 18.3, and 8.6 mJ/m$^2$, respectively, which were about half of those between water and the respective probe liquids.
Table A.2 The measured surface and interfacial tensions.

<table>
<thead>
<tr>
<th>Test liquid</th>
<th>Surface tension and its components (mJ/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
</tr>
<tr>
<td>Water</td>
<td>73.0±0.3</td>
</tr>
<tr>
<td>30 % sucrose</td>
<td>74.4±0.4</td>
</tr>
<tr>
<td>3.5 % salt</td>
<td>73.5±0.3</td>
</tr>
<tr>
<td>PBS</td>
<td>71.8±0.3</td>
</tr>
<tr>
<td>ES cell medium</td>
<td>64.4±1.2</td>
</tr>
</tbody>
</table>

Figure A.3 Surface tension of aqueous solutions measured in air using the pendant drop method under a humidity control environment. The surface tension of water measured is about 73.0 mJ/m² for the entire experiment. For the 30 wt % sucrose solution, the surface tension was 74.4 mJ/m². For the ES cell medium, a quick reduction in surface tension is observed from 0 (~ 66 mJ/m²) to about 200s after the drop is formed, and then it maintains at a stable value of ~ 51.0 mJ/m² for the rest of the duration (up to 20 min).
Table A.3 Surface tensions and their components of aqueous solutions estimated using different combinations of probe liquids.

<table>
<thead>
<tr>
<th>Test liquid</th>
<th>Combinations of probe liquids</th>
<th>Surface tension and its components (mJ/m²)</th>
<th>( \gamma^\text{tot} )</th>
<th>( \gamma^\text{LW} )</th>
<th>( \gamma^\text{AB} )</th>
<th>( \gamma^+ )</th>
<th>( \gamma^- )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>BA-EA-EE</td>
<td>73.3</td>
<td>21.9</td>
<td>51.4</td>
<td>26.0</td>
<td>25.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BN-EA-EE</td>
<td>73.3</td>
<td>21.9</td>
<td>51.3</td>
<td>25.9</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC-EA-EE</td>
<td>73.2</td>
<td>21.9</td>
<td>51.3</td>
<td>25.8</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC-BN-EE</td>
<td>73.1</td>
<td>21.8</td>
<td>51.3</td>
<td>25.8</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reported(^{123,124})</td>
<td>72.8</td>
<td>21.8</td>
<td>51.0</td>
<td>25.5</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td>30 % sucrose</td>
<td>BA-EA-EE</td>
<td>81.4</td>
<td>22.8</td>
<td>58.6</td>
<td>34.9</td>
<td>24.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BN-EA-EE</td>
<td>77.4</td>
<td>27.2</td>
<td>50.3</td>
<td>24.3</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC-EA-EE</td>
<td>77.1</td>
<td>27.6</td>
<td>49.5</td>
<td>23.5</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC-BN-EE</td>
<td>74.8</td>
<td>25.0</td>
<td>49.8</td>
<td>23.2</td>
<td>26.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reported(^{206})</td>
<td>85.9</td>
<td>25.56</td>
<td>60.34</td>
<td>25.5</td>
<td>36.12</td>
<td></td>
</tr>
<tr>
<td>3.5 % salt</td>
<td>BA-EA-EE</td>
<td>78.4</td>
<td>22.1</td>
<td>56.4</td>
<td>31.5</td>
<td>25.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BN-EA-EE</td>
<td>76.9</td>
<td>23.7</td>
<td>53.2</td>
<td>27.5</td>
<td>25.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC-EA-EE</td>
<td>76.5</td>
<td>24.1</td>
<td>52.4</td>
<td>26.6</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC-BN-EE</td>
<td>74.0</td>
<td>21.5</td>
<td>52.5</td>
<td>26.3</td>
<td>26.2</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>BA-EA-EE</td>
<td>79.6</td>
<td>28.1</td>
<td>51.5</td>
<td>26.7</td>
<td>24.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BN-EA-EE</td>
<td>79.1</td>
<td>28.7</td>
<td>50.4</td>
<td>25.4</td>
<td>24.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC-EA-EE</td>
<td>78.5</td>
<td>29.3</td>
<td>49.2</td>
<td>24.2</td>
<td>25.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC-BN-EE</td>
<td>74.9</td>
<td>25.2</td>
<td>49.7</td>
<td>23.7</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td>ES cell medium</td>
<td>BA-EA-EE</td>
<td>64.7</td>
<td>35.6</td>
<td>29.1</td>
<td>7.6</td>
<td>27.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BN-EA-EE</td>
<td>63.2</td>
<td>37.6</td>
<td>25.6</td>
<td>5.8</td>
<td>28.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC-EA-EE</td>
<td>63.0</td>
<td>37.8</td>
<td>25.1</td>
<td>5.5</td>
<td>28.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OC-BN-EE</td>
<td>61.6</td>
<td>36.0</td>
<td>25.6</td>
<td>5.5</td>
<td>30.0</td>
<td></td>
</tr>
</tbody>
</table>
Figure A.4 shows some representing interfacial tensions as a function of time. With octane (Figure A.4a) as the probe liquid, the $\gamma_{\text{water/OC}}$ was $\sim 51$ mJ/m$^2$, as expected, for the entire measurement period. For the sucrose solution, the interfacial tension decreased slightly (from $\sim 50.5$ mJ/m$^2$ to $\sim 49.9$ mJ/m$^2$) during the first few seconds, and then maintained at $\sim 48.4$ mJ/m$^2$ throughout the rest of the experiment. For the cell culture medium, the interfacial tension started out at $\sim 27.4$ mJ/m$^2$ and quickly reduced to about 20 mJ/m$^2$ in $\sim 200$ s, and then maintained at this value.

When ethyl acetate was the probe liquid (Figure A.4b), $\gamma_{\text{water/EA}}$ was $6.8$ mJ/m$^2$ initially and then increased slightly (likely due to the high solubility of water in ethyl acetate that caused the shape of the water drop to change). For the sucrose solution, the first measured interfacial tension was $7.1$ mJ/m$^2$. It fluctuated slightly for over a period of $100$ s and then decreased (to $\sim 6.5$ mJ/m$^2$) until the drop was detached. For the cell culture medium, the initial interfacial tension was $6.1$mJ/m$^2$. During the first $25$ s, a fast decreasing trend was observed, and then the interfacial tension continued to decrease slowly from $\sim 4$ mJ/m$^2$ (at $30$ s) to $\sim 1.5$ mJ/m$^2$ (at $600$ s).

As shown in the case of ethyl acetate, the solubility of probe liquids in water could cause errors in the interfacial tension measurement. To rule out or account for this possibility, the interfacial tension measurement using water-saturated ethyl acetate (Figure A.4c) was carried out and compared to the data obtained using pure ethyl acetate (Figure A.4b), and the trends of interfacial tension as a function of time were found to be quite different. For water, the measured interfacial tension using water-saturated ethyl
acetate was 6.7 mJ/m$^2$, similar to that measured using pure ethyl acetate, and this value maintained for the entire measurement period (20 min). For the sucrose solution, the interfacial tension measured was ~ 7.8 mJ/m$^2$ throughout the entire duration. The drop of the sucrose solution formed in the water-saturated ethyl acetate appeared to be stable and doesn’t shrink and then detached from the tip of needle as that found when using pure ethyl acetate. For the cell culture medium, the interfacial tension is 6.4 mJ/m$^2$ at time 0, followed with a continuous decreasing trend to a value of ~ 3.7 mJ/m$^2$ at 50 s, and maintained at ~ 3.7 mJ/m$^2$ for the rest of the experiment.

Comparing Figure A.4b and c, only slight difference in initial interfacial tensions is noticed due to the saturation of water in ethyl acetate. More obvious differences are observed for the trends of interfacial tensions for sucrose solution and culture medium as the contact time between the two liquid phases increases. This could be the result of water in the aqueous solution (i.e. the drop), migrates into ethyl acetate, in the case of the pure ethyl acetate, leading to an increase solute concentration in the drop. As a result, the drop shrinks, and as in the case of the culture medium, the interfacial tension continues to decrease, or as in the case of 30 wt % sucrose solution, the drop detaches due to its small size. Nevertheless, the results from this experiment indicate that a probe solvent with a high water solubility might only affect the interfacial tension measurement after water in a solution has the time to migrate to the probe liquid and resulting in an increase of the solution concentration, the interfacial tension measured right after the drop is formed still closely represents the expected value resulted from a sharp water/oil interface.
Figure A.4 The dynamic interfacial tension. (a) At the water-octane interface: $\gamma_{\text{water/OC}} = \sim 51 \text{ mJ/m}^2$, $\gamma_{\text{sucrose/OC}} = \sim 49.9 \text{ mJ/m}^2$ (decreased slightly from $\sim 50.5 \text{ mJ/m}^2$ during few seconds), and $\gamma_{\text{medium/OC}}$ is initially $\sim 27.4 \text{ mJ/m}^2$ and it quickly reduces to about 20 mJ/m$^2$ in $\sim 200$ s, and then maintains at this value. (b) At the water-ethyl acetate (purified) interface: $\gamma_{\text{water/EAC}}$ is initially $6.8 \text{ mJ/m}^2$ and the trend of the interfacial tension shows a slight increase. $\gamma_{\text{sucrose/EAC}}$ is firstly measured $7.3 \text{ mJ/m}^2$. It slightly fluctuates for over a period of 100 s and then decreases until the drop is detached. $\gamma_{\text{medium/EAC}}$ is initially $6.1 \text{ mJ/m}^2$, and during the first 25 s, a fast decreasing trend is observed, and then maintained at this value. (c) At the water-ethyl acetate (saturated with water): $\gamma_{\text{water/EAC}}$ is initially $6.7 \text{ mJ/m}^2$. $\gamma_{\text{sucrose/EAC}}$ is initially measured $7.8 \text{ mJ/m}^2$ and stable. $\gamma_{\text{medium/EAC}}$ is initially $6.4 \text{ mJ/m}^2$. Stiff decreasing trend of the interfacial tension is continued for 25 s and it becomes stable for the entire experiment.
A.3.3 Using Interfacial Tensions to Obtain Surface Tensions and Its Components of Aqueous Solutions.

The liquid-liquid interfacial tensions reported in Table A.2 are used to estimate the surface tension components using equation (3). The results are summarized in Table A.3. For water, all combinations lead to similar surface tensions in a range 73.3 ~ 73.1 mJ/m², and the LW and AB components of ~ 21.8 mJ/m² and ~ 51.4 mJ/m², respectively. All these values are very close to the values reported in literatures, indicating the method of liquid-liquid interfacial tensions is applicable to determine surface tension and its components of water.

For other aqueous solutions, the surface tensions and their components appear to depend on which probe liquid combination was used. Surface tensions of 30 wt % sucrose, for example, are 81.4, 77.4, 77.1, 74.8 mJ/m², respectively, using the combination of BA-EA-EE, BN-EA-EE, OC-EA-EE, and OC-BN-EE. The highest value is resulted from the combination (BA-EA-EE) that has the lowest interfacial tensions with water. The same trend is observed for the other three aqueous solutions. The large difference (~ 6.5 mJ/m²) in the total surface tension values determined from the four combinations is observed for 30 wt % sucrose solution and 3.5 wt % salt solution; while the difference is rather trivial for PBS and the cell culture medium. The LW and AB components also vary, but not in a clear trend. For sucrose solution and cell culture medium, the more concentrated aqueous solutions, the $\gamma^{LW}$ from BA-EA-EE (i.e. the combination with the lowest interfacial tensions) appears to be lower than the values obtained using the other three combinations. For the dilute aqueous solutions, 3.5 wt %
salt and PBS, no large difference in $\gamma^{\text{LW}}$ for the four combinations of probe liquids is observed. The highest $\gamma^{\text{AB}}$ is noticed to be from the combination of BA-EA-EE, while the other three combinations provide similar values. When comparing to water, most values for 30 wt % sucrose solution, 3.5 wt % salt solution, PBS are higher. Cell culture medium has dramatic lower values of surface tension ($62 - 65 \text{ mJ/m}^2$) and its AB component ($25 - 29 \text{ mJ/m}^2$) from those of water, while the LW component ($35 - 38 \text{ mJ/m}^2$) is much higher.

Both inorganic solutes (i.e. NaCl) have higher surface energy than that of water, it is expected that surface tension of their aqueous solutions to be higher than that of water. For the 30 % (w/w) sucrose solution, the estimated surface tension components and parameters, by Docoslis et al. based on the contact angle measurement on dried state of sucrose and its solubility in water, are also presented in Table A.3. Our total surface tension values determined from the measured liquid-liquid interfacial tensions were all lower. According to Docoslis et al., the increased total surface tension of the sucrose solution is mainly due to the increase in $\gamma^{\text{AB}}$: i.e. $\Delta \gamma^{\text{LW}} = 3.76 \text{ mJ/m}^2$ and $\Delta \gamma^{\text{AB}} = 9.34 \text{ mJ/m}^2$ ($\Delta \gamma_i = \gamma^{\text{sucrose}}_i - \gamma^{\text{water}}_i$ with $i$ being LW, AB, or tot). In our measurement, a comparable total surface tension with the values of Docoslis et al. was obtained by BA-EA-EE combination, and the increase is due to the large increase in $\Delta \gamma^{\text{AB}} = 7.6 \text{ mJ/m}^2$ with the small increase in $\Delta \gamma^{\text{LW}} = 1.0 \text{ mJ/m}^2$. For results from the other combinations, the opposite trend that the large increase of $\Delta \gamma^{\text{LW}}$ (5.4, 5.8, and 3.2 mJ/m², respectively for $\Delta \gamma^{\text{LW}}$ of BZ-EA-EE, OC-EA-EE, and OC-BZ-EE) with slight changes in $\Delta \gamma^{\text{AB}}$ (–1.4 ~ 0.7 mJ/m²) was found. This different trend should be from the components of the
probe liquids. In the case of the use of octane, which has only an apolar component, combinations of OC-EA-EE and OC-BN-EE estimated the large increase of $\Delta \gamma_{\text{LW}}$ with the small changes of $\Delta \gamma_{\text{AB}}$. However, as the use of benzaldehyde, which has a high $\gamma_{\text{LW}} = 40.0$ mJ/m$^2$ but also has a high $\gamma^\ominus = 14.0$ mJ/m$^2$, the combination of BA-EA-EE estimated a high $\gamma_{\text{AB}} = 58.6$ mJ/m$^2$. Additionally, unless benzene is a mono-polar liquid as like benzaldehyde, it estimated similar trend with the case of octane. It might be due to its relatively small $\gamma_{\text{AB}} \approx 2.7$ mJ/m$^2$) than its $\gamma_{\text{LW}}$ (28.9 mJ/m$^2$). While the result from this study indicates that the use of probe liquids forming lower interfacial tensions with water does not completely eliminate migration of molecules from/to the interface due to the system trying to reduce its energy; such migration is likely lessened to result in the surface tension that is closer to the estimated value as compared to using probe liquids that form higher interfacial tensions with water. Compared to the components of $\gamma^\oplus$ and $\gamma^\ominus$ of Docoslis et al. and ours, quite different values are found. It could be resulted from two effects, first, experimental error due to the mutual solubility of organic liquids in water, and second, the interactions with polar moieties of organic liquids and sucrose at the interface.

For 3.5 wt % of sodium chloride solution, the surface tension is estimated as 78.4 mJ/m$^2$ by a combination of BA-EA-EE and 76.9, 76.5, and 74 mJ/m$^2$ are estimated, respectively for BZ-EA-EE, OC-EA-EE, and OC-BZ-EE. In the case of BA-EA-EE, similar to sucrose, the increase of $\gamma_{\text{tot}}$ (i.e. $\Delta \gamma_{\text{tot}} = 5.6$ mJ/m$^2$) as compared to water is mainly due to the increase of $\gamma_{\text{AB}}$ (i.e. $\Delta \gamma_{\text{AB}} = 5.4$ mJ/m$^2$) by using mono-polar probe liquids, the increase of $\gamma_{\text{LW}}$ is only about 0.3 mJ/m$^2$. As we discussed above, the trend of
increased $\Delta \gamma^{LW}$ and $\Delta \gamma^{AB}$ by utilizing octane or mono-polar liquids (BA, EA, and EE) was also found in the estimated surface tension and its components of the 3.5 % of salt solution. To the best of our knowledge, we couldn’t find any reported value about surface tension and components of salt solutions that can be compared with. It is conjectured that the significant increase of the surface tension, by using a combination of organic liquids, might be due to the attraction between the ions ($\text{Na}^+$ and $\text{Cl}^-$) in water and the polar site (e.g. carbonyl group) of the organic molecule, which oriented towards the water-organic liquid interface.

Our determined surface tension of PBS is the highest surface tension among all test liquids. Compared to water, the large increase is mainly due to the significantly higher $\gamma^{LW}$ term than that of water. Compared to 3.5 wt % salt solution, it is quite different results since the increase of the salt solution is mainly due to the large increase of $\gamma^{AB}$ term. For PBS, $\gamma^{AB}$ term is similar to that of water. Currently the exact reasons are unclear.

For the cell culture medium, due to the presence of amphiphilic proteins that can assemble at the water/oil interface, a reduction of the surface and interfacial tension is expected. Also, most hydrated proteins (e.g. serum albumin) have a $\gamma^{LW}$ of $\sim$28 mJ/m$^2$, therefore, the determined values using the liquid-liquid interfacial tensions are reasonable.
A.4. Conclusions

Surface tension and its components of aqueous solutions are determined by using liquid-liquid interfacial tensions of various combinations of organic probe liquids. We hypothesize that using probe liquids having lower interfacial tension with water, the driving force for solutes to migrate away from or towards the water-oil interface can be reduced, thus resulting more accurate values determined using the method of liquid-liquid interfacial tensions. Five probe liquids, representing a wide range (6 ~ 50 mJ/m²) of interfacial tension with water are used. Of those, four combinations of three probe liquids each are selected, based on sensitivity analysis, to determine surface tensions and their components of five test aqueous solutions. The surface tension and its components of water (reference I) determined using all four combinations agree well with reported values. With the use of combination composed of lower interfacial tensions, the highest surface tensions were estimated by the reduction of solutes interaction at water-oil interface. As a result, surface tensions of 30 wt % of sucrose solution, 3.5 wt % of salt solution, PBS, ES cell medium were estimated as high as 81.4, 78.4, 79.6, and 64.7 mJ/m². It is also necessary to mention that the method might present two limitations. First, using probe liquids that form lower interfacial tensions with water not necessarily eliminate all interactions at the interface. Second, the choice of organic liquids affects the final results of the estimated surface tension and its components. Nevertheless, more accurate surface tensions, as compared to those measured in air, can be resulted by applying the liquid-liquid interfacial tension method, especially using probe liquids that form low interfacial tensions with water.
APENDIX B

ESTIMATION OF MAXIMUM NUMBER OF ROLLING OF BILAYER FILMS

$R_{eq}$ can be estimated from equation (19). Using the estimated $R_{eq}$, the maximum number ($N_{\text{max}}$) of rolling of bilayer films from each side can be estimated.

By assuming that the bilayer films roll tightly without any gaps in between each layers and the tubes are not deformed, the length of circumferences for the first rolling, the second rolling, and the maximum rolling ($N_{\text{max}}$) are expressed as $2\pi(R_{eq} + h)$, $2\pi(R_{eq} + 2h)$, and $2\pi(R_{eq} + N_{\text{max}}h)$, respectively. The maximum rolling is achieved when the summation the circumferences of the bilayer films is equal to $0.5L - (R_{eq} + N_{\text{max}}h)$. Therefore, the above equations can be summarized as,

$$0.5L - (R_{eq} + N_{\text{max}}h) = \sum_{n=1}^{N_{\text{max}}} 2\pi(R_{eq} + nh)$$

(A.4)

and it becomes,

$$0.5L = \sum_{n=1}^{N_{\text{max}}-1} 2\pi(R_{eq} + nh) + 2\pi(R_{eq} + N_{\text{max}}h) + (R_{eq} + N_{\text{max}}h)$$

(A.5)

To write it as $N_{\text{max}}$,

$$N_{\text{max}} = \frac{0.5L - (2\pi + 1)R_{eq} - \sum_{n=1}^{N_{\text{max}}-1} 2\pi(R_{eq} + nh)}{(2\pi + 1)h}$$

(A.6)
Figure A.5 Schematics of initial bilayer films (light gray color, width: $L$, thickness: $h$) on substrate and rolled films (light green: PDLA, dark green: PLGA) with equilibrium radius ($R_{eq}$).