‘BREATH FIGURE’ PLGA FILMS AS IMPLANT COATINGS FOR
CONTROLLED DRUG RELEASE

AN ABSTRACT

SUBMITTED ON THE TWENTY FIFTH DAY OF APRIL 2013

TO THE DEPARTMENT OF CHEMICAL AND BIOMOLECULAR ENGINEERING
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
OF THE SCHOOL OF SCIENCE AND ENGINEERING
OF TULANE UNIVERSITY
FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY

BY

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ABSTRACT

The breath figure method is a versatile and facile approach of generating ordered micro and nanoporous structures in polymeric materials. When a polymer solution (dissolved in a high vapor pressure organic solvent) is evaporated out in the presence of a moist air stream, the evaporative cooling effect causes the condensation and nucleation of water droplets onto the polymer solution surface. This leads to the formation of an imprinted porous structure upon removal of the residual solvent and water. The facile removal of the water droplet template leaving its structural imprint is a specifically appealing aspect of the breath figure film technology.

The first part of the dissertation work involves the fabrication of drug loaded breath figure thin films and its utilization as a controlled drug release carrier and biomaterial scaffold. In a single fabrication step, single layer/multilayer porous thin films were designed and developed by combining the breath figure process and a modified spin or dip coating technique. Using biodegradable polymers such as poly (lactic-co-glycolic acid) (PLGA) and poly (ethylene glycol) (PEG), drug loaded films were fabricated onto FDA approved medical devices (the Glaucoma drainage device and the Surgical hernia mesh). The porosity of the films is in the range of 2-4 µm as characterized by scanning electron microscope. The drug coated medical implants were characterized for their surface and bulk morphology, the degradation rate of the film, drug release rate and cell cytotoxicity. The results suggest that the use of breath figure morphologies in biodegradable polymer films adds an additional level of control to drug release. In comparison to non-porous films, the breath figure films showed an increased degradation and enhanced drug release. Furthermore, the porous nature of the film was investigated as
a biomaterial scaffold to construct three dimensional *in vitro* tissue model systems. The breath figure film with interconnected pores facilitates cell infiltration and tissue remodelling *in vitro*, suggesting its high potential in regenerative medicine and tissue engineering applications.

In the second part of the dissertation, the versatility of breath figure polymers was explored as a reverse template to create micropatterned soft materials. Unlike traditional lithographic masters, the breath figure assembly is a simple and cost-effective approach to create micro/nano sized “bead” like uniform patterns on the surface of hydrogels and biopolymers. By incorporating iron nanoparticles into the pores, this technique was extended to form hydrogels decorated with nanoparticles specifically in the pattern. The morphology features and the functional characteristics were demonstrated through scanning electron microscopy. The potential applications of these micro-fabricated materials in biosensors and cell culture substrates are outlined.
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS.......................................................................................................................... ii

LIST OF TABLES...................................................................................................................................... viii

LIST OF FIGURES..................................................................................................................................... ix

CHAPTERS

1. Introduction........................................................................................................................................ 1

1.1 Breath Figures.................................................................................................................................. 1

1.2 Preparation and Mechanism.............................................................................................................. 1

1.3 Significance....................................................................................................................................... 4

1.4 Polymeric Drug Delivery Systems..................................................................................................... 5

1.5 Hypothesis and Specific Aims........................................................................................................... 7

2. In Vitro Degradation and Release Characteristics of Spin Coated Thin Films of PLGA with a “Breath Figure” Morphology............................................................ 10

2.1 Summary......................................................................................................................................... 10

2.2 Introduction..................................................................................................................................... 11

2.3 Materials and Methods.................................................................................................................... 13

2.4 Results and Discussion..................................................................................................................... 19

2.5 Conclusions..................................................................................................................................... 33

3. A Novel Antiproliferative Drug Coating for Glaucoma Drainage Devices................................. 34

3.1 Summary......................................................................................................................................... 34

3.2 Introduction..................................................................................................................................... 35
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3 Materials and Method</td>
<td>38</td>
</tr>
<tr>
<td>3.4 Results and Discussion</td>
<td>45</td>
</tr>
<tr>
<td>3.5 Conclusions</td>
<td>63</td>
</tr>
<tr>
<td>4. Fabrication and <em>In Vitro</em> Characterization Of A Breath Figure Based Antimicrobial Coating For Biological Surgical Meshes</td>
<td>64</td>
</tr>
<tr>
<td>4.1 Summary</td>
<td>64</td>
</tr>
<tr>
<td>4.2 Introduction</td>
<td>65</td>
</tr>
<tr>
<td>4.3 Materials and Methods</td>
<td>67</td>
</tr>
<tr>
<td>4.4 Results and Discussion</td>
<td>72</td>
</tr>
<tr>
<td>4.5 Conclusions</td>
<td>89</td>
</tr>
<tr>
<td>5. A Novel Three-dimensional PLGA Scaffold System Using The Breath Figure Method For <em>In Vitro</em> Evaluation Of Mammary Morphogenesis And Chemotherapy Response</td>
<td>90</td>
</tr>
<tr>
<td>5.1 Summary</td>
<td>90</td>
</tr>
<tr>
<td>5.2 Introduction</td>
<td>91</td>
</tr>
<tr>
<td>5.3 Materials and Methods</td>
<td>94</td>
</tr>
<tr>
<td>5.4 Results and Discussion</td>
<td>106</td>
</tr>
<tr>
<td>5.5 Conclusions</td>
<td>129</td>
</tr>
<tr>
<td>6. A Novel Micropatterning Of Hydrogels Using Breath Figures As Template</td>
<td>130</td>
</tr>
<tr>
<td>6.1 Summary</td>
<td>130</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

6.2 Introduction ................................................................. 131
6.3 Materials and Methods .................................................. 134
6.4 Results and Discussion .................................................... 137
6.5 Conclusions ..................................................................... 154

**GLOSSARY** ………………………………………………………………………………….. 155

**LIST OF REFERENCES** ............................................................. 156
## LIST OF TABLES

<table>
<thead>
<tr>
<th>No.</th>
<th>Table Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Values of the exponent $n$ and rate constant $k^*$ for all polymer films. $R^2$ is the regression coefficient</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>Primer sequences, Melting temperature (Tm), and expected amplicon sizes in bp</td>
<td>104</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Schematic illustration shows the simple fabrication of breath figures by exposing the polymer solution to the humid air</td>
<td>02</td>
</tr>
<tr>
<td>2</td>
<td>Degradation pathways of PLGA polymer</td>
<td>06</td>
</tr>
<tr>
<td>3</td>
<td>Chemical Structure of the polymers and drug components</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>Mechanism of incorporation of a drug into thin PLGA film by ‘breath figure’ and spin coating technique</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>Scanning Electron Microscopy (SEM) of breath figure PLGA film</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>High magnification SEM images of breath figure PLGA film</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>SEM of breath figure PEG/PLGA film</td>
<td>24</td>
</tr>
<tr>
<td>8</td>
<td><em>In vitro</em> degradation pattern of breath figure PLGA film</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td><em>In vitro</em> degradation pattern of breath figure PEG/PLGA film</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td><em>In vitro</em> release kinetics of breath figure films</td>
<td>30</td>
</tr>
<tr>
<td>11</td>
<td>Scanning Electron Microscopy Characterization of bare silicone and PLGA film coated silicone</td>
<td>46</td>
</tr>
<tr>
<td>12</td>
<td>Dose response of Mitomycin C and its in-vitro release from the surface of single layer porous PLGA film</td>
<td>49</td>
</tr>
<tr>
<td>13</td>
<td>Schematic illustration of 5-FU incorporation into the double-layered breath figure PLGA films with and without MMC</td>
<td>53</td>
</tr>
<tr>
<td>14</td>
<td><em>In vitro</em> release characteristics of 5-FU from the double layered breath figure PLGA films (Formulation I)</td>
<td>55</td>
</tr>
<tr>
<td>15</td>
<td><em>In vitro</em> cytotoxicity of 5-FU loaded double layered breath figure PLGA films from Formulation I.</td>
<td>57</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td><em>In vitro</em> cytotoxicity of 5-FU/double layered breath figure PLGA films from Formulation II</td>
<td>59</td>
</tr>
<tr>
<td>17</td>
<td>The change in morphology of the 0.4 mg 5-FU loaded double-layered PLGA film incubated for a period of 28 days.</td>
<td>61</td>
</tr>
<tr>
<td>18</td>
<td>Chemical structure of the Vancomycin</td>
<td>67</td>
</tr>
<tr>
<td>19</td>
<td>Schematic illustration of the anti-microbial coating on surgical mesh using the breath figure PLGA thin film morphology</td>
<td>73</td>
</tr>
<tr>
<td>20</td>
<td>(A) Photograph of Surgisis® mesh (15 cmx10cm). (B) Scanning Electron Micrograph (SEM) showing the herringbone groove pattern on the surface</td>
<td>74</td>
</tr>
<tr>
<td>21</td>
<td>SEM images of the Surgisis® mesh coated with the breath figure PLGA film</td>
<td>75</td>
</tr>
<tr>
<td>22</td>
<td>Release kinetics of vancomycin from the breath figure PLGA and PEG/PLGA films</td>
<td>78</td>
</tr>
<tr>
<td>23</td>
<td>SEM micrographs showing degradation of breath figure PLGA and PEG/PLGA films coated on the surgical mesh for 28 days incubation</td>
<td>80</td>
</tr>
<tr>
<td>24</td>
<td>Kirby-Bauer Analysis for control samples indicating the zone of inhibition (ZOI) data as a function of time</td>
<td>82</td>
</tr>
<tr>
<td>25</td>
<td>Kirby-bauer analysis for breath figure PLGA coated mesh containing Vancomycin</td>
<td>84</td>
</tr>
<tr>
<td>26</td>
<td>Kirby-bauer plot for breath figure drug loaded PEG/PLGA film coated mesh</td>
<td>87</td>
</tr>
<tr>
<td>27</td>
<td>Experimental set up illustrates the fabrication of breath figure PLGA and cell culture on these porous scaffolds <em>in vitro</em></td>
<td>97</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>Scanning electron microscopy (SEM) images of spin coated porous and non-porous PLGA films</td>
<td>108</td>
</tr>
<tr>
<td>29</td>
<td>Morphological analysis of MCF-7 cells cultured for 7 days on 2D controls and 3D spin coated PLGA</td>
<td>110</td>
</tr>
<tr>
<td>30</td>
<td>SEM of MCF-7 cells cultured on 2D systems and 3D spin coated PLGA</td>
<td>112</td>
</tr>
<tr>
<td>31</td>
<td>Low and high magnification SEM images of dip coated porous PLGA and the comparison of percentage population of pores measured for both spin coated and dip coated samples</td>
<td>113</td>
</tr>
<tr>
<td>32</td>
<td>Low and high magnification of morphological development of MCF-7 cells cultured on dip coated porous PLGA</td>
<td>116</td>
</tr>
<tr>
<td>33</td>
<td>3D scaffolds support more robust lobulo-alveolar growth with or without mammary differentiating agents</td>
<td>119</td>
</tr>
<tr>
<td>34</td>
<td>Expression of genes involved in mammary cell differentiation and some stem cell markers is enhanced in cells grown on 3D PLGA scaffolds in the presence or absence of differentiating agents</td>
<td>123</td>
</tr>
<tr>
<td>35</td>
<td>Growth proliferation kinetics of MCF-7 cells on 3D dip coated, 3D spin coated and 2D glass cover slips</td>
<td>125</td>
</tr>
<tr>
<td>36</td>
<td>Doxorubicin treatment for MCF-7 cells grown on 2D control and 3D PLGA scaffolds</td>
<td>128</td>
</tr>
<tr>
<td>37</td>
<td>The fabrication of micro-patterned PHEMA hydrogel surface using breath figure polymer as reverse template</td>
<td>138</td>
</tr>
<tr>
<td>38</td>
<td>Scanning electron microscopy images of breath figure PLGA film and the corresponding PHEMA pattern from the PLGA film</td>
<td>140</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>Scanning electron microscopy images of breath figure PEG/PLGA template and patterned hydrogel using breath figure film as reverse template</td>
<td>143</td>
</tr>
<tr>
<td>40</td>
<td>Confocal and SEM analysis of whole sample (hydrogel and PEG/PLGA template)</td>
<td>146</td>
</tr>
<tr>
<td>41</td>
<td>The schematic diagram and SEM of double-side patterned PHEMA hydrogels</td>
<td>148</td>
</tr>
<tr>
<td>42</td>
<td>The schematic illustration (A) of filling iron nanoparticles into the breath figures and placing of iron nanoparticles at the protrusion of hydrogel. B) The SEM images of breath figure PEG/PLGA template filled with Fe nanoparticles uniformly.</td>
<td>149</td>
</tr>
<tr>
<td>43</td>
<td>The micro-patterned biopolymers obtained using the reverse template</td>
<td>152</td>
</tr>
</tbody>
</table>
This Dissertation is dedicated to

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My Siblings (Thirumaran and Kalaivani)
Chapter 1 - Introduction

1.1 Breath figures

The term “breath figures” is synonymous to fogging and named to describe the patterning of condensed water droplets on the cold surface\(^1\)-\(^3\). Ordered arrays of porous structure are formed when a polymer solution is brought in contact with humid gas stream. The main requisite in the formation of breath figure arrays is that the polymer has to be dissolved in highly volatile, water-immiscible, high-vapor pressure organic solvents (Chlorinated solvents, toluene, benzene, carbon disulphide etc.)\(^4\)-\(^5\). The breath figure method produces pores in the range of 0.2 \(\mu\)m to 20 \(\mu\)m, which depends on the structure of the polymers and other process parameters such as humidity, temperature of the system, concentration and volume of polymer solution\(^6\).

1.2 Preparation and Mechanism

A simple fabrication procedure is illustrated in Figure 1. When the polymer solution is dropped on the substrate and exposes to humid air, the polymer solution turns quickly into an opaque film as the solvent evaporates out. The opaque nature of the film is because of the emulsification of water droplets that condense on the polymer surface. Eventually, the dried film contains uniformly arranged imprint of water droplets (porous cavities). The breath figure approach is feasible in the laboratory, as it does not require sophisticated instrumentation and it can be performed using a single step process.
Figure 1. Schematic illustration shows the simple fabrication of breath figures by exposing the polymer solution to the humid air.

The processing and the mechanism of formation of breath figures pores can be explained as various steps. a) When the moist air flow is directed across the polymer solution, the rapid evaporation of the solvent results in decrease in temperature on the polymer surface. It has been reported in various studies that this cooling of the polymer surface results in temperatures below room temperature and in some cases even below zero degree celsius. The cooling effect on the surface induces the condensation of water droplets from the atmospheric humidity and initiates the nucleation of water droplets on the polymer surface. This step is called evaporation cooling effect. b) Following nucleation, large number of water droplets condenses and grows as a function of time. The temperature difference between the imperfectly packed water droplets on the surface induces thermo capillary convection. Laterally acting capillary forces and convective
motion resulting from these temperature gradients on the solution surface, stabilizes the water droplets. The stabilization helps to arrange the water droplets in an ordered manner without coalescing them\(^4\), \(^7\). This effect is called Marangoni Convection. c) Increase in time means increase in viscosity and the solidification of polymer. The solidifying polymer surrounds the packed water droplets and encapsulates to form porous architecture. Once the film is sufficiently dried after removal of residual solvent and water, the film with orderly arranged porous structure is formed. The parameters that control the pore formation and its size distribution are humidity, polymer concentration, casting volume and temperature of the system.

Humidity is one of the major parameters that control the pore size and distribution. In general, the pore size can be increased with the increase of humidity as more water droplets are available for the nucleation and growth\(^6\). However, a critical range of humidity (between 60% and 90% relative humidity) is necessary to obtain uniform and monodisperse pore size. An excessively low humidity (< 30% RH) impairs water condensation while a very high humidity results in the formation of pores with broad range of sizes.

A variety of polymers have been successfully used to form the porous materials. Polymer concentration in the casting solution can be tuned to control the pore size and thickness of the dried film. Increasing the polymer concentration results in smaller pores and thicker walls. Volume of the polymer solution can also be varied to control the pore size. The pore size depends on the size of the water droplet templates that forms after nucleation and growth, and the water droplets size depend on the solvent evaporation time, which is equivalent to the water droplets condensation time. Therefore, the bigger
the casting volume of polymer solution, the longer the water condensation time and this allows the water droplet to grow larger which leads to the formation of larger pore size. The temperature in the system during breath figure process is another important controlling parameter of pore size. The substrate can be either cooled or decrease the temperature of the humid chamber prior to the casting, to suppress the evaporation of solvent. Subsequently, a longer solvent evaporation time leads to bigger water droplets and therefore, bigger pore size\(^6,10\).

1.3 Significance

Ordered porous structures using the breath figures have attracted many researchers because of their potential application in microelectronics (photonic band gaps and optical stop-bands), membranes (separation of materials), super-hydrophobic surfaces, antireflection coatings, catalysis and sensors\(^{11, 12}\). Moreover, polymers with multilayered porous structures present a major interest in biomedical applications such as drug delivery systems, cell culture substrates, tissue engineering and placing biomolecules selectively in the pores\(^{13, 14}\). In comparison to other conventional methods (colloidal crystals and emulsions) to create porous structures, the breath figure method offers several advantages. The breath figure technique does not require any templating material to create pores in the polymer. Instead, water droplets act as template and self-removal of these water templates is the main attraction in the breath figures and the entire processing is faster, and highly reproducible.
1.4 Polymeric Drug Delivery Systems

Controlled release systems using biodegradable and non-biodegradable synthetic polymers as drug carriers have been studied extensively for biomedical and healthcare application\(^{15}\). The controlled release system offers several advantages in comparison to traditional drug delivery systems as the controlled release strategy provides improved safety, enhanced therapeutic efficiency and minimal side effects. The major limitation of using non-biodegradable polymers is the removal of device/polymeric implant after the drug has depleted completely from the polymer. This effects the patient compliance and puts an economic burden for the secondary surgery to remove the implant. On the other hand, drug delivery using biodegradable polymers offer significant advantages because the implant devices or polymer can be designed in such a way that the polymer eventually absorbs into the body eliminating the possibility of any toxic effects or secondary surgery\(^{16}\).

Among all biodegradable polymers studied in the last two decades, Poly (lactic-co-glycolic acid) (PLGA) has been the focus of extensive research in drug delivery\(^{15}\) systems and has also been approved by FDA for clinical use\(^{16,17}\). PLGA is synthesized by catalytic reaction based on ring opening polymerization with lactide and glycolide as starting cyclic dimers\(^{18}\), \(^{19}\). It is completely biodegradable\(^{18-20}\) and biocompatible and undergoes hydrolytic degradation to release oligomers and monomers (lactic acid and glycolic acid). The monomers are then easily eliminated from the body as CO\(_2\) and H\(_2\)O as it passes through tricarboxylic acid metabolic cycle (Figure 2). They have high biocompatibility, low immunogenicity and induce less toxic effects to the body.
Figure 2. Degradation pathways of PLGA polymer in aqueous media
1.5 Hypothesis and Specific aims

In the biomedical applications, PLGA polymer has been used in a variety of forms\textsuperscript{18, 21, 22} such as microspheres, nanoparticles, films, tablets, scaffolds etc. Most of these forms are prepared either using emulsion process or solvent casting procedure and very few studies\textsuperscript{23} have been undertaken to prepare PLGA with a microporous structure using the breath figure process. The main goal of this work was to synthesize porous and thin PLGA films using the breath figure methodology and apply these films as coatings, scaffolds and templates for various biomedical applications. Hypothesis is that the presence of micro-structured pores in the PLGA will increase the drug release when it is used as coatings for medical implants.

Three potential applications of the breath figure PLGA films were proposed and studied as a part of the research. The first application is to utilize the films as coatings on medical implants and control the release of the pharmaceutical drugs locally at the implant site. Such coatings provide multiple effects at the implant site. Firstly, the desired drug release kinetics and therapeutic level can be achieved by altering the properties or composition of the polymers used. The large surface area rendered by porous nature of the film will also accelerate the degradation of the film, resulting into macro porous structures, which then act as scaffolds to promote neovascularization\textsuperscript{24}. Moreover, the films can be stacked with multiple layers of PLGA loaded with both hydrophilic and hydrophobic drug components with the breath figure morphology.

In the second application, the porous nature of the breath figure was exploited to act as an extracellular matrix to facilitate cell adhesion and proliferation. The feasibility of constructing a three dimensional \textit{in vitro} tissue model was explored, by varying pore
sizes of the breath figures in the range of 2 microns to 30 microns.

As a third potential application, the versatility of the breath figure polymer films was explored in the micro-fabrication technology. The honeycomb-like structured breath figure pores was used as a master template to create monolayer bead-like patterns on soft materials such as biopolymers and hydrogels. Unlike traditional lithographic techniques (Photolithography, Soft lithography, Colloidal templates etc), the breath figure based approach offers various advantages: single step fabrication, more flexible, economical and convenient method to create patterns on water soluble soft materials with high fidelity.

In order to achieve the above-mentioned proposals, the following specific aims were evaluated.

Specific Aim (1) - In the second chapter of dissertation, breath figure PLGA films were synthesized and incorporated with model drug components, Ibuprofen and Salicylic acid, into thin films. The drug loaded breath figure films were characterized for morphological features, in vitro degradation and release kinetics and compared the results with the non-porous films.

Specific Aim (2) - In the third chapter, we designed a novel double-layered PLGA films with porous structure using the breath figure method. The films loaded with antifibrotic drugs were fabricated specifically for preventing fibrosis in the post-surgical period of Ahmed Valve glaucoma surgery. Highly stable drug, 5-Fluorouracil was incorporated into the bottom PLGA layer and the less stable Mitomycin C was dispersed into the top PLGA layer and these films were examined for the release pattern controlled by diffusion and degradation of PLGA and correlated with in vitro cell cytotoxicity study.
Specific Aim (3) - In the fourth chapter, a single layer breath figures loaded with an antibiotic drug was coated on the surgical hernia mesh. Again, the films were analyzed for prolonged duration of drug release (4 weeks) and correlated the in vitro tests with the bacteria inhibition analysis.

Specific Aim (4) - In the fifth chapter, different pore size ranges of breath figure PLGA scaffolds were synthesized using modified spin and dip coating procedures. Using MCF-7 cells (breast cancer epithelial cell line) as model cell line, the scaffolds were cultured for a week to construct three dimensional function of cells in vitro and compared the results with the two dimensional controls.

Specific Aim (5) - Sixth chapter explores the versatility of the breath figure films by using these films as a template to prepare micro-patterning on hydrogels surface and biopolymers. The morphological characteristics of the formation of hydrogel patterns and functional characteristics were studied. The novelty of this technique is highlighted and its potential application in drug delivery, tissue engineering and biosensors.
Chapter 2 - *In Vitro* Degradation And Release Characteristics Of Spin Coated Thin Films Of PLGA With A “Breath Figure” Morphology

2.1 Summary

Poly (lactic-co-glycolic acid) (PLGA) coatings on implant materials are widely used in controlled drug delivery applications. Typically, such coatings are made with non-porous films. This study describes a synthesis of thin PLGA film coating with a highly ordered microporous structure using a simple and inexpensive water templating breath figure technique. A single stage process combining spin coating and breath figure process was used to obtain drug incorporated porous thin films. The films were characterized by scanning electron microscope (SEM) to observe the surface and bulk features of porosity and also, degradation pattern of the films. Moreover, the effect of addition of small amount of poly (ethylene glycol) (PEG) into PLGA was characterized. SEM analysis revealed an ordered array of ~ 2 µm sized pores on the surface with the average film thickness measured to be 20 µm. The incorporation of hydrophilic poly (ethylene glycol) (PEG) enhances pore structure uniformity and facilitates ingress of water into the structure. A five week *in vitro* degradation study showed a gradual deterioration of the breath figure pores. During the course of degradation, the surface pore structure deteriorates to initially flatten the surface. This is followed by the formation of new pinprick pores that eventually grow into a macro porous film prior to film breakup.
Salicylic acid (highly water soluble) and Ibuprofen (sparingly water soluble) were chosen as model drug compounds to characterize release rates, which are higher in films of the breath figure morphology rather than in non-porous films. The results are of significance in the design of biodegradable films used as coatings to modulate delivery.

2.2 Introduction

Poly (lactic-co-glycolic acid) (PLGA), a biodegradable and biocompatible FDA approved polymer, is being increasingly used in sustained drug delivery applications\textsuperscript{25-30}. The polymer undergoes hydrolytic degradation under physiological conditions breaking down into poly (lactic acid) and poly (glycolic acid) and eventually to lactic and glycolic acid, which are easily eliminated by tricarboxylic acid metabolic pathways\textsuperscript{16, 31}. PLGA coatings on implant materials such as stents\textsuperscript{27} allow a slow release of drug with the coating eventually being degraded away. Typically, such coatings are made with thin films that are solvent cast on the support material\textsuperscript{27, 29, 32, 33}. Such films have very low porosities and after the initial burst of surface bound drug, further release occurs primarily through the degradation of the polymer\textsuperscript{33}.

The present study describes the fabrication of very thin films with breath figure porous structures of PLGA and correlates the drug delivery characteristics of such films with the details of degradation. Breath figure polymer morphologies were first pioneered by Srinivasarao and coworkers as a simple method to fabricate ordered arrays of pores in a polystyrene film\textsuperscript{4}. In this technique, the polymer is dissolved in a low boiling organic solvent and cast under humid conditions resulting in the formation of an ordered pore structure on the film surface\textsuperscript{5, 34-36} with a dense under layer. Briefly, the mechanism of
pore formation involves the rapid evaporation of the solvent that causes a cooling effect on the polymer solution surface. The cooling induces the condensation of water droplets (from humid air) onto the solution surface. Micron-sized water droplets nucleate on the surface and subsequently grow to form the arrays of ‘islands’ that eventually produces the breath figure pattern. These arrays do not coalesce, but penetrate into the polymer solution, which acts as a substrate for subsequent condensation and nucleation of water droplets. The polymer film forms around water droplet/solution interface and encapsulates the water droplets preventing coalescence. Locally acting lateral capillary forces and convective motion resulting from temperature gradients on the solution surface stabilize the water droplets arranging in an ordered manner\textsuperscript{37, 38}. Once the film is dried at room temperature, the evaporation of residual solvent and water leads to the formation of a surface patterned with a microporous structure. The breath figure process is simple, economically viable and easily reproducible\textsuperscript{23, 39-42}.

Because of the highly porous nature of breath figures, it is possible that release characteristics of encapsulated pharmaceutical compounds can be significantly modified in comparison to nonporous films of the same material. It is therefore important to characterize such release especially in connection with the fact that the polymer film degrades with time. The present study describes the synthesis of drug-loaded porous PLGA films prepared using the breath figure method and attempts to correlate the release kinetics to the degradation characteristics. A single stage process combining spin coating and the breath figure technique was used to obtain drug incorporated porous thin films. In addition to PLGA, PEG was used as a plasticizer to modify pore structures and release characteristics through incorporation of a hydrophilic polymer into the film. Salicylic
acid and Ibuprofen were used as model drugs compounds. Salicylic acid is a water soluble compound (solubility >2 mg/ml) while ibuprofen has a limited solubility in water (solubility <0.5 mg/ml). Pioneering work by Wang and coworkers have shown morphological progressions in the degradation of relatively thick nonporous biodegradable films with correlation to the lag phase in drug release \(^{43}\). However, the work described in the current study is the first instance of correlating time-dependent breath figure morphology changes, to release characteristics. Potential applications of this study include the design of very thin coatings on medical devices and surgical implants where release profiles can be designed through control of surface structures and/or sandwich type layers.

### 2.3 Materials and Methods

#### 2.3.1 Materials

Poly (D,L-lactide-co-glycolide) (PLGA 50:50) (Figure 3A) polymer (Resomer RG 504 \(M_w=56000\) & Inherent viscosity = 0.56 dl/g) was purchased from Boehringer Ingelheim Chemicals Inc, USA. Methylene chloride (organic solvent - ACS grade) was obtained from Fisher Scientific, USA. Teflon sheets, 0.2 mm thick, used as substrate materials, were purchased from Scientific Commodities Inc. (catalog no: BB9558). All other chemicals, Poly(ethylene glycol)(PEG- Average \(M_w=3350\) g/mol) (Figure 3B), Ibuprofen (≥ 98% GC) (Figure 3C), Salicylic acid (99%) (Figure 3D), Disodium hydrogen phosphate (Na\(_2\)HPO\(_4\)), Potassium dihydrogen phosphate (KH\(_2\)PO\(_4\)), Sodium Chloride (NaCl), and Potassium Chloride (KCl) were obtained from Sigma Aldrich Chemicals. All chemicals were used as received, without further purification.
2.3.2 Synthesis of “breath figure” PLGA polymer films

A spin coater (model WS-400-6NPP-LITE, Laurell Technologies Corporation, North Wales, PA) was used to prepare the film. A 1.5 cm square piece of teflon, used as the substrate was rinsed with 95% ethanol to remove any surface contaminants. The substrate was then placed on the spin table which is connected to a vacuum to hold the substrate while spinning. The coating chamber is connected to a flow of humid air created by bubbling the air through the distilled water. Although the humidity can be modified by mixing the air with dry nitrogen and in all the experiments, relative humidity was maintained at about 70% as measured by a hygrometer (Fisher Scientific).

Figure 4 illustrates the entire process of breath figure thin film fabrication. To prepare the coating solution, 1% (w/v) of the drug (Ibuprofen or Salicylic acid) was first dissolved in methylene chloride followed by dissolving 15% (w/v) of the PLGA polymer. The solution was vortex mixed to ensure homogeneity. The appropriate volume (0.4 mL) of the solution containing PLGA and the drug (67 µg drug/mg polymer) was dropped onto the substrate and the spinning process was immediately accelerated to 2500 rpm for 30-45 sec. During the spin coating process, the solvent evaporates to form an opaque film. The coated films were dried for at least a day at room temperature. In experiments with PEG, the ratio of PEG to PLGA was 1:9. Similar procedures were followed to prepare control breath figure films without the drug component. To prepare non porous films, the gas supply was switched from humid air to dry nitrogen. All films were easily peeled off from the teflon substrate. To clearly obtain release and morphological characteristics from breath figure coated systems, the films were reattached to teflon squares using double-sided tape (3M). This ensures that both release and degradation will
occur primarily from the porous surface of the film. Verification of drug content in the polymer film was done by redissolving the thin polymer film in an excess of dichloromethane, removing the solvent (to create very small particles of the polymer) to precipitate the drug and then extracting the drug into phosphate buffered saline solution (PBS) for analysis.

**Figure 3.** Chemical Structure of the polymers and drug components used. (A) Poly(lactic-co-glycolic acid)(PLGA) (B) Poly(ethylene glycol)(PEG) (C) Ibuprofen (D) Salicylic acid
Figure 4. Mechanism of incorporation of a drug into thin PLGA film by ‘breath figure’ and spin coating technique
2.3.3 Characterization and analysis of breath figure polymer film

2.3.3.1 Film morphology

Morphological characterizations of all films were done using a field emission scanning electron microscopy (FE-SEM; Hitachi S-4800) at an accelerating voltage of 3 kV. The films were mounted on the SEM sample holder and gold coated using a sputter coater (Polaron SEM coating system) set at 20 mA for duration of 75 sec. All films were imaged in the dry state which is appropriate for PLGA films which in contrast to hydrogels, do not absorb water significantly and therefore do not change morphology. A minimum of three films were examined to characterize surface and bulk morphologies.

2.3.3.2 In vitro degradation of breath figure polymer film

Experiments to understand the in vitro degradation of breath figure PLGA and PEG/PLGA films were done at 37 °C in phosphate buffered saline solution (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Sodium Phosphate dibasic and 2 mM Potassium Phosphate monobasic). The pH was then adjusted to 7.4 using 0.1M HCl. The films coated on teflon were suspended and incubated in the buffer solution for 35 days, and subjected to slow stirring using a magnetic stir bar. The PBS medium was changed every week to maintain constant pH. Each week, a small piece of polymer film was cut from the original film, rinsed carefully with distilled water and dried at room temperature for at least a day prior to imaging. The experiments were conducted in triplicate.
2.3.3.3 Surface contact angle measurements

The wettability of breath figure films was measured using the sessile drop method with a standard goniometer (Rame-Hart model 250) and analyzed using the DROPimage Advanced software for contact angle determination. A 3 μL distilled water droplet was placed on the polymer film surface and the contact angle ‘θ’ measured. The measurement was done for a minimum of 5 samples of a specific polymer film, and the average value reported. Typical standard deviations are of the order of 0.3.

2.3.3.4 In vitro release characteristics

Ibuprofen and Salicylic acid were used as model drugs to characterize the release profiles of breath figure polymer films. The equivalent non-porous smooth films were used as controls. In vitro release studies were carried out by incubating 1.5 cm side square drug incorporated films in 15 ml of PBS medium at 37 °C and stirred gently using a magnetic stirrer. At specific time intervals, 0.650 ml aliquots of the solution was withdrawn and centrifuged to remove any possible debris from the degrading polymer. Then, the aliquot was returned to the vial after measuring the absorbance to quantify drug release. The pH of the medium was monitored during the course of the experiment to verify that the solution is buffered adequately during polymer degradation. Ibuprofen and salicylic acid release were quantified through the absorbance at 221 and 296 nm, respectively. Standard calibration plots of ibuprofen and salicylic acid absorbance were constructed to correlate absorbance with drug release levels. All experiments were conducted in triplicate.
2.4 Results and Discussion

2.4.1 Characterization of “breath figure” patterned thin polymer films

Figures 5A and 5B show morphological details of breath figure PLGA films in comparison to the nonporous films obtained by spin coating in a dry atmosphere (shown as corresponding insets to the figures). The SEM micrograph in Figure 5A reveals an ordered array of approximately 2 µm sized pores on the surface, observed over a large surface area. The pores dimensions created from PLGA are reproducible and simple to create. Figure 5B is an oblique view of the cross section and the surface and reveals both surface features and aspects of bulk porosity. Clearly, the pore structure is prevalent almost throughout the film but ends in a dense bottom layer of around 2 µm thickness. The average thickness of the film measured by a micrometer is 20 µm. The insets to Figures 5A and 5B reveal corresponding morphologies of a spin coated PLGA film without incorporating the breath figure technique. As observed, the film clearly lacks the surface and bulk porosity characteristics of breath figures. Figures 6A and 6B reveal higher magnification top and oblique view images of the PLGA breath figure film. After peeling off the film from the teflon substrate, the dense bottom layer was imaged showing the lack of any discernible pores (inset to Figure 6B).
Figure 5. Scanning Electron Microscopy (SEM) of breath figure PLGA film

(A) Low magnification of surface topography (Inset: Surface morphology of non-porous PLGA film)

(B) Low magnification of cross-sectional view (Inset: Cross-sectional morphology of non-porous PLGA film)

We have used the analysis described by Bolognesi and coworkers\textsuperscript{44}, to understand pore penetration in the bulk polymer film. As these authors have stated, pore formation can be described through the minimization of the free energy at the three phase (water droplet, air, polymer solution) interface, with a dimensionless pore penetration $Z_0 = Z/R$ where $Z$ is the distance of the droplet center from the air-solvent interface and $R$ is the droplet radius. $Z_0$, the value of $Z$ at which the free energy is minimized is expressed as,

\[
Z_0 = \frac{\gamma_w - \gamma_{w/s}}{\gamma_s} \tag{1}
\]

where $\gamma_w$ and $\gamma_s$ are the surface tensions of the air-water interface and the air-solvent interface, respectively, and $\gamma_{w/s}$ is the interfacial tension between water and the solvent. For values $-1 < Z_0 < 1$, the water droplets will be located at the interface between
air and solution with partial exposure to both fluids. Upon formation of the final breath figure morphology, such systems will only consist of a single layer of pores below which is a dense nonporous layer. For $Z_0$ values greater than unity, the droplets will penetrate below the surface, the consequence of which is a multi-layered porous polymer structure. For the PLGA-methylene chloride system, $Z_0$ is 1.62, based on the air-water surface tension, $\gamma_w$ (72.8 dynes/cm), the air-methylene chloride surface tension, $\gamma_s$ (28.12 dynes/cm) and the water-methylene chloride interfacial tension, $\gamma_{w/s}$ (27.2 dynes/cm). The deep penetration of pores in the PLGA system studied here is due to the penetration of water droplets below the solvent-air interface. Figure 6C shows the droplet shape for contact angle determination of breath figure PLGA, from which a value of 81° was obtained, indicating the relative hydrophobicity of the material.
Figure 6. High magnification SEM images of breath figure PLGA film
(A) Surface Morphology (B) Cross-sectional morphology (Inset: surface morphology of dense bottom layer)
(C) Contact angle of breath figure PLGA film
Poly (ethylene glycol) (PEG) is a well-studied plasticizer for PLGA and as a hydrophilic material is expected to increase hydrophilicity of breath figure films. We have found that PEG incorporation leads to a much better definition of pore structure and an enhanced hydrophilicity. Figure 7 illustrates the influence of PEG addition at a 1:9 ratio of PEG to PLGA. The pore structure is highly monodisperse with the pores arranged in ordered hexagonal arrays, as shown in Figures 7A and 7B. The addition of small quantity of PEG improves the degree of ordering and orientation of pores. Again, we see the deep penetration of pores into the polymer film (Figure 7C) with an almost row-by-row arrangement. Our experiments indicate that the optimal PEG incorporation level is approximately 10 wt% and when PEG is added to values greater than 15 wt %, the film tends to become patchy. Figure 7D indicates that the PEG incorporated breath figure has enhanced hydrophilicity as shown by a contact angle of 67°. Imaging software (Image-Pro Plus version 5.0) was used to measure pore size distributions. A minimum of three SEM images were used with the dimensions of 200 pores measured to obtain statistics on pore size distributions. The average pore diameters are 1.4 µm and 2.5 µm for the breath figure PLGA and PEG/PLGA films, respectively and the corresponding standard deviations are 0.296 µm and 0.668 µm.

The incorporation of breath figure morphologies significantly adds to surface areas of the film. For example, if the surface pores are assumed to be hemispheres, the surface area occupied by each pore ($2\pi r^2$) is double that of the equivalent flat surface ($\pi r^2$). Assuming hexagonal order in the surface pores, with a pore to pore distance ‘a’ (center to center distance of $2r+a$), it is derived that the fractional increase in surface area per unit cell on the film surface is $(\pi r^2/(2r+a)^2\cos(\pi/6))$ which translates into an increase
of 58% with pores of radius, $r = 1 \, \mu m$ and pore to pore distances, $a = 0.5 \, \mu m$ as example dimensions. With the deep penetration of pores into the breath figure films, it is expected that release rates would be further enhanced as the polymer gradually degrades exposing underlying pores.

![A](image1)

![B](image2)

![C](image3)

![D](image4)

**Figure 7.** SEM of breath figure PEG/PLGA film
(A) and (B) Low and High magnification of surface morphology
(C) Cross-sectional morphology
(D) Contact angle of breath figure PEG/PLGA film
2.4.2 In vitro degradation of breath figure films

2.4.2.1 Degradation of PLGA films

Figure 8 illustrates the morphological characteristics of the degradation of the breath figure film over a period of 35 days. To simulate an environment where the film is used as a coating for medical devices, we examined films coated on a teflon substrate so that the degradation is primarily through the porous breath figure surface. Within 7 days, clear morphological changes are observed with the deterioration of the top layer of pore walls leading to a flatter topology as shown by the arrow in Figure 8A (day 7). As degradation proceeds, intervening ridges between the pores become less distinct as the surface layers are degraded. The surface pores eventually become depressions on the surface of the film as the ridges dissolve away. The side view (Figure 8B, day 7) also illustrates that the film has significantly decreased in thickness to 5-10 µm. With time the pores in the lower layers of the original film become revealed as the polymer surface continues to degrade (box in Figure 8A, day 14). We note however that as degradation proceeds to the vicinity of the originally dense non porous bottom layer, small submicron pinprick pores are generated that are not part of the original breath figure structure. The pinprick pores are denoted by the arrows in the micrographs denoting day 21 degradation characteristics. The number density of these pinprick pores increases (after 28 days) and the film begins to break up. In 35 days, cracks and islands of macro porous film remnants are observed. The surface of the film also becomes wrinkled. We find that throughout the degradation process, the film continues to adhere to the teflon substrate.
Figure 8. *In vitro* degradation pattern of breath figure PLGA film
(A) Surface morphology
(B) Cross-sectional morphology
2.4.2.2 Degradation of PEG/PLGA films

Figures 9A and 9B show the effect of PEG in the degradation of PLGA film. Essentially the same progression of deterioration is observed with an initial surface flattening, the exposure of underlying pores, and the eventual formation of new pinprick pores that grow and eventually rupture the film. The addition of PEG may increase the degradation rate as we observe the initial morphological change of underlying pores coming into view is apparent within 7 days of degradation.

These observations are useful as the sequence of events that lead to film breakup through degradation is not evident. It is interesting that the initial pores of the breath figure do not propagate into larger pores leading to rupture. Rather, there is an initial flattening of surface topography and then the emergence of new underlying pores.
Figure 9. *In vitro* degradation pattern of breath figure PEG/PLGA film
(A) Surface morphology
(B) Cross-sectional morphology
2.4.3 Release characteristics of breath figure polymer films

The release profile of breath figure PLGA and PEG/PLGA films was carried out in PBS medium (pH 7.4, 0.1M), incubated at 37 °C. A non-porous film with the equivalent amount of dispersed drug was used as control. The choice of salicylic acid as a model drug component is due to its high water solubility (>2 mg/mL) and clearly measurable UV absorbance at 296 nm. Figure 10A illustrates the cumulative release of salicylic acid from the PLGA film and PEG/PLGA film respectively, for a period of 8 days. The release illustrates the effects of breath figure morphology and the role of incorporating PEG into the film. We note that within 3 hours, differences between the breath figures and the corresponding non porous films, and between PLGA and PEG/PLGA films are observed, and the differences become more pronounced at later times. All films with the breath figure morphology show a higher release rate than the corresponding non breath figure, and the PEG/PLGA films show increased release rates compared to direct PLGA films. We also note that most of the release is completed within 5 days during which there are changes in surface topography, but no formation of pinprick pores and breakdown of the film.
Figure 10. *In vitro* release kinetics of breath figure films
(A) Release profile of Salicylic acid from PLGA and PEG/PLGA films
(B) Release profile of Ibuprofen from PLGA and PEG/PLGA films
The situation is similar in the case of ibuprofen (Figure 10B) although release takes place over a longer period which involves the formation of pinprick pores during the latter release stages and the gradual initiation of film breakdown. Again, breath figure morphologies indicate faster release than the corresponding nonporous analogs. However, we do see that the incorporation of PEG in the nonporous film enhances release over the pure PLGA breath figure indicating the strong role played by PEG in allowing water ingress.

In thin films where release is through Fickian diffusion, \( \frac{M_t}{M_\infty} \) the fractional release can be expressed as \(^{43, 46}\)

\[
\frac{M_t}{M_0} = 4 \left( \frac{Dt}{\pi l^2} \right)^{\frac{1}{2}} = kt^{\frac{1}{2}}
\]

the traditional formulation for transient diffusion in a slab at small values of the dimensionless time \( \left( \frac{Dt}{\pi l^2} \right) \), where D is the diffusivity, l, the slab thickness and k a lumped parameter\(^{46}\). In situations when release is entirely through dissolution of the polymer matrix, the release rate is independent of time, therefore leading to

\[
\frac{M_t}{M_0} = k't
\]

One can therefore write a heuristic expression

\[
\frac{M_t}{M_0} = k'^{*}t^n
\]

where \( n \) values close to 0.5 indicate diffusion as the primary release mechanism while \( n \) values close to unity indicate degradation as driving release. Values of \( k'^{*} \) and \( n \)
are listed in Table 1 for both systems. The significant deviation from the exponent of unity in the correlation for both salicylic acid and ibuprofen release implies that transport through diffusion dominates over release due to the degradation of the polymer. With such thin films, we do not see an extended lag phase that would indicate the secondary phase where degradation becomes the primary driver for release. Even with the formation of pinprick pores initiating the film degradation process during the release period for ibuprofen, thin films of such materials allow encapsulated species to diffuse relatively small distances to the film surface for release with diffusion being the primary mode of release.

<table>
<thead>
<tr>
<th>Polymer films</th>
<th>Salicylic acid</th>
<th>Ibuprofen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>k^*</td>
</tr>
<tr>
<td>Breath figure PLGA</td>
<td>0.32</td>
<td>0.78</td>
</tr>
<tr>
<td>Non-porous PLGA</td>
<td>0.34</td>
<td>0.75</td>
</tr>
<tr>
<td>Breath figure PEG/PLGA</td>
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<td>0.85</td>
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<td>Non-porous PEG/PLGA</td>
<td>0.29</td>
<td>0.81</td>
</tr>
</tbody>
</table>

**Table 1.** Values of the exponent n and rate constant k^* for all polymer films. R^2 is the regression coefficient.
2.5 Conclusions

Morphological characteristics of breath figure films of degradable PLGA and PEG/PLGA materials were analyzed through scanning electron microscopy as they were allowed to degrade in vitro. The degradation pattern shows a flattening of surface structure where the walls of the surface breath figure pores are first degraded away, followed by the gradual degradation of the underlying layers. Pinprick pores extending to the base of the film are subsequently formed which evolve into larger pore structures that eventually break up the film.

The morphology of the film has a significant effect on release characteristics with breath figure morphologies in general exhibiting faster release than their nonporous analogs. Additionally the incorporation of poly (ethylene glycol) into the films enhances release rates which we attribute to improvement of water ingress into the film. Drug release from such thin films appears to follow diffusion pathways rather than a constant release rate based on degradation of the material through dissolution of surface layers.

The use of breath figure morphologies in biodegradable polymer films adds an additional level of control to drug release. Coating medical devices (stents, surgical meshes etc.) with thin biodegradable films allows exploitation of controlled release without significant modifications of the mechanical properties of the substrate materials. For example, coated surgical meshes need to retain the highly flexible nature of the underlying synthetic or tissue based material. To utilize the breath figure films in in-vivo applications, further investigation is needed by applying the coatings directly into the medical devices.
Chapter 3 - A Novel Antiproliferative Drug Coating for Glaucoma Drainage Devices

3.1 Summary

The implantation of a glaucoma drainage device (GDD) is often necessary for intractable cases of glaucoma. Currently, the success rate of GDD implants is relatively low because fibrosis that develops during the wound healing process ultimately blocks fluid drainage. This study describes a novel porous coating for Ahmed glaucoma valves based on biodegradable poly (lactic-co-glycolic acid) (PLGA). The creation of a layered coating facilitates a burst of mitomycin C (MMC) release followed by a slow release of 5-fluorouracil (5-FU), which together prevent fibrosis over the most active period of postoperative wound healing (0-28 days). Thin films of PLGA were fabricated using a spin-coating technique. The procedure led to an asymmetric pore structure that was exploited to control the rate of dissolution. Double-layered porous films were constructed to achieve continuous drug release. A cell culture system was used to test the efficacy of these coatings. 5-FU was stable when incorporated directly into PLGA films, while MMC was stable only when surface-loaded. Double-layered films containing 5-FU only in the bottom layer showed a 3-5 day delay in drug release, followed by a sharp increase that continued for ~28 days. Continuous release of antifibrotics was achieved by using a double-layered PLGA structure with 5-FU incorporated into the bottom layer and MMC surface-loaded onto the top layer.
The combined use of both MMC and 5-FU in a biodegradable device has the potential to reduce fibrosis and increase the success rate of GDD implants. The design is simple and can be scaled for commercial production.

3.2 Introduction

Reduction and control of the intraocular pressure (IOP) is the mainstay of treatment in the management of glaucoma. Elevated IOP can be reduced pharmacologically via daily eye drops, or surgically by trabeculectomy and/or implantation of a glaucoma drainage device (GDD). The Tube versus Trabeculectomy (TVT) study has shown that there are advantages to using GDDs to control intraocular pressure, including the shunting of aqueous humor to the posterior subconjunctival space, as opposed to the anterior subconjunctival space\textsuperscript{47, 48}. Data from the recently completed 5-year TVT study may lead to an earlier and wider use of GDD for the treatment of glaucoma. What currently limits enthusiasm for GDDs is the development of inner wall bleb fibrosis, which hampers outflow facility and increases intraocular pressure. The success rate of GDD implants is \(~70\%\) to \(80\%\) at one year and \(40\%\) to \(50\%\) five years postoperatively because of the development of fibrosis\textsuperscript{49}. A GDD that could regulate flow without the development of fibrosis could potentially become a first line treatment for glaucoma.

The antimetabolites 5-fluorouracil (5-FU) and mitomycin C (MMC) have been used for many years to attenuate the wound healing response after trabeculectomies\textsuperscript{50}; however, the administration of these drugs intraoperatively during Ahmed valve surgery has not improved surgical outcomes \textsuperscript{51}. More recent reports have demonstrated that the
adjunctive use of MMC and 5-FU both intraoperatively and postoperatively (one injection into the bleb/week for 5 weeks) resulted in a higher long-term success rate, a lower rate of the hypertensive phase, and a lower rate of complications\textsuperscript{52, 53}. Because application of antimetabolites at the time of surgery can be imprecise, and injection into the bleb can result in unacceptable discomfort and risk of infection for the patient, there is a clear need for drug delivery systems that can be installed intraoperatively to deliver antifibrotic drugs during the period of wound healing that occurs after GDD implantation.

A slow-release form of 5-FU in a modified Baerveldt valve has been reported\textsuperscript{54}. In this study, a collagen plug containing 5-FU was placed into the drainage tube and implanted into rabbit eyes. Eyes with drains containing 5-FU had significantly lower IOP and maintained this lower pressure for a longer time period. However, by the end of the study (13 weeks) IOP had returned to preoperative conditions for all groups and the differences between capsule thickness in the control and experimental groups, although significant, were relatively modest (~20%). Our laboratory has developed a drug-loaded, p(HEMA)-modified Ahmed valve that released MMC into the bleb for the first ~10 days after implantation and led to much larger decreases in capsule thickness\textsuperscript{55, 56}. In this prototype, however, the P(HEMA) polymer used for drug delivery remained attached to the valve and there were concerns that this non-biodegradable polymer might lead to long-term complications. A biodegradable polymer based on butyric acid (polyhydroxyalkanoates) has also been proposed as a drug delivery drainage system\textsuperscript{57}. In this report, polymerized polyhydroxyalkanoate was used to manufacture the drainage tube of an aqueous shunt. These shunts were subsequently dip- or spray-coated with anti-proliferative and anti-inflammatory pharmaceuticals (paclitaxel and triamcinolone...
Acetonide) and drug release into saline was measured over a 10-day period. No measures of the efficacy of the released drug were presented. The stent material itself showed some cytotoxicity to ocular cells in culture (5-50%, depending upon polymerization conditions) and degraded in saline over a period of ~12 months. The authors hypothesized that once the drainage tube had degraded, tissue remodeling would result in a drainage channel that no longer required physical support by a polymer tube. Their preliminary in vivo experiments showed that the tubes induced moderate neovascularization in the cornea and conjunctiva of implanted rabbits at 100 days after surgery; but the sample size was low (n=1) and no significant conclusions could be drawn from these experiments.

In the present study, we describe a simple and scalable procedure to fabricate a thin drug-containing polymer coating on commercially available Ahmed valves. These coatings were designed to deliver the antifibrotic agents (mitomycin C and/or 5-fluorouracil) into the subconjunctival space and inhibit postoperative fibrosis. The method involves the creation of films of “breath figure” porous poly (lactic-co-glycolic acid) (PLGA) with incorporated drugs. The porous structures can be advantageous in modifying the drug release characteristics and the degradation pattern of the polymer. To achieve a relatively continuous release of anti-fibrotic activity over a 20-30 day period, we have fabricated double-layered porous PLGA films, in which the more stable 5-FU is dispersed into the bottom layer and the top layer is surface-loaded with MMC. The morphology of these films, and their ability to release anti-proliferative drugs and the efficacy of the drugs released from the films using in vitro cytotoxicity assays are the subject of this study.
3.3 Materials and Methods

3.3.1 Materials

Poly (D,L-lactide-co-glycolide) (PLGA 50:50) polymers, Resomer RG 504 (M_w=56,000; Inherent viscosity = 0.56 dl/g) and Resomer RG 506 (M_w = 96,000; Inherent viscosity = 0.80 dl/g) were purchased from Boehringer Ingelheim Chemicals Inc., (Petersburg, VA). Methylene chloride (ACS grade) was obtained from Fisher Scientific, USA. Ahmed glaucoma valves (Model FP7) and the medical grade silicone sheets from which these valves were manufactured were generous gifts from New World Medical Inc. (Rancho Cucamonga, CA). Mitomycin C (MMC) (derived from Streptomyces caespitosus), 5-fluorouracil (5-FU), neutral buffered formalin and toluidine blue were from Sigma Aldrich Chemicals (St Louis, MO). Transwells® with 12mm 0.4µm pore polyester membrane inserts and 12-well Costar tissue culture plates were purchased from Corning (Wilkes Barre, PA). COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in a humidified atmosphere of 5%CO_2/95% air in glutamine-free DMEM (4500g/L glucose and 1.5g/L sodium bicarbonate) supplemented with 10% fetal bovine serum, 4mM glutamine, 1mM sodium pyruvate, 100IU/mL penicillin, 100µg/mL streptomycin and 0.25µg/mL amphotericin B. Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Dulbecco’s Modified Eagle’s Medium (DMEM), L-glutamine, antibiotic-antimycotic solution, and sodium pyruvate were from Life Technologies (Grand Island, NY). All chemicals were used as received, without further purification.
3.3.2 Coating of medical grade silicone with the “breath figure” PLGA films

A spin coater (model WS-400-6NPP-LITE, Laurell Technologies, North Wales, PA) was used to prepare thin PLGA films with a microporous structure. Using a trephine, discs were cut from medical grade silicone sheets identical to those used in the manufacture of the Ahmed valves. The discs were rinsed with distilled water and 95% ethanol to remove any surface contaminants. The discs were then placed on the spin turntable, which was subsequently connected to a vacuum line to hold the substrate in place while spinning. In order to maintain high relative humidity, the spin coating chamber was connected to a flow of humid air created by bubbling the air through distilled water. A relative humidity of at least 70% (measured using a hygrometer) was maintained in the chamber during all spin coatings. The films were dried at room temperature.

A preliminary experiment was performed to determine the optimum concentration of MMC to use in subsequent experiments with COS-1 cells. In the presence of humid air, 20 µL of PLGA solution (15% RG 504 w/v in methylene chloride), was loaded onto 3.5 mm silicone discs and spin coated (2500 rpm for 30 sec) to produce the breath figure PLGA films. After the films had dried, MMC was dissolved in a solvent mixture (5:1 v/v methylene chloride : tetrahydrofuran) and dropped uniformly onto the surface of breath figure PLGA films. After drying, the samples were UV sterilized for an hour prior to conducting studies of drug release and COS-1 sensitivity to the released drug. The cytotoxicity of PLGA films loaded with three different levels of MMC (0.25, 1.0 and 5 µg) were evaluated (described below). Control samples containing no MMC were prepared in an identical manner.
3.3.3 Formulation I: 5-FU loaded double-layered breath figure PLGA films

The 5-FU drug particles were ground into a fine powder using a mortar and pestle set. The appropriate weight of finely ground drug was then dispersed into the PLGA solution (12.5% RG 506 w/v in methylene chloride) using bath sonication for 10 minutes. This resulted in a homogeneous “milky” polymer-drug solution. Under humid conditions, 75 µL of the polymer-drug solution was spun onto a washed 8 mm silicone disc for ~ 6 minutes at 200 rpm. This low speed spin insured that all of the polymer solution remained on the substrate. Three different loadings of 5-FU samples were prepared (0.1, 0.4 and 2 mg per 8 mm disc). The samples were dried for a day before fabricating a second layer. To coat a second layer, 50 µL of RG 504 PLGA (15% w/v in methylene chloride) was spin coated at 1000 rpm for 25 s. This process created a very thin film with the average thickness of 20 microns and was intended to be a seal over the first layer. All samples were UV-sterilized before studies of in vitro release, cell cytotoxicity and polymer degradation.

3.3.4 Formulation II: 5-FU + MMC – loaded double-layered breath figure PLGA

In Formulation II, double-layered films were fabricated as described for Formulation I, then MMC was surface-loaded into the top layer. Based on preliminary dose response results, 1 µg of MMC was chosen for Formulation II. After fabrication of the double-layered films, 1µg MMC in solution (5:1 v/v methylene chloride : tetrahydrofuran) was dispersed on the PLGA surface while reproducing the breath figure in a manner very similar to that used in the preliminary studies described above. To compare the effect of added MMC, three different loadings of 5-FU (0.1, 0.4 and 2 mg
per 8 mm disc) were prepared, each with 1 µg of MMC surface loaded on the top of the PLGA film.

3.3.5 Morphology of breath figure PLGA films

The morphology of uncoated silicone and breath figure PLGA-coated samples was characterized using field emission scanning electron microscopy (Hitachi S-4800) as previously described\textsuperscript{58}. All samples were coated with a thin layer of gold using a sputter coater (Polaron SEM coating system) prior to imaging. Both the pore dimensions and the thickness of coatings were examined. Samples containing 0.4 mg 5-FU were chosen for a study of the degradation pattern of double-layered 5-FU loaded PLGA films. These samples were incubated in 10 mL PBS (pH, 7.4) at 37°C for periods up to 28 days. At weekly intervals, films were withdrawn, rinsed carefully with distilled water, and then air-dried prior to imaging.

3.3.6 Kinetics of drug release from PLGA breath figure films

Release studies on films loaded with 1 µg and 5 µg MMC were performed in glass vials containing 1 mL PBS as the release medium. This experiment was not performed for polymer samples containing 0.25 µg MMC, due to the difficulty in quantifying UV absorbance at this low drug concentration. Aliquots (0.65 mL) of the PBS were removed at specific time intervals after immersion of the polymer samples, and absorbance was measured at 364 nm using a UV spectrophotometer (Shimadzu UV-1700 series). After measurement, the aliquots were returned to the vials to maintain constant
volume and sink conditions. The concentration of drug release was calculated from a linear calibration curve plotted from known concentrations of MMC.

Release studies on films loaded with 5-FU were performed in a similar manner. In these experiments, 8 mm discs containing 5-FU incorporated films were incubated in 10 mL of PBS at 37°C. At specific time intervals, a 1 ml aliquot of the solution was withdrawn and 1 ml of fresh PBS was added to the vial to maintain constant volume. The pH of the medium was monitored during the course of the experiment to verify that the solution was buffered adequately during polymer degradation. Each aliquot was centrifuged to remove any possible debris from the degraded polymer components that could interfere with the absorbance readings and drug release was quantified through the absorbance measured at 266 nm. A standard calibration plot of 5-FU absorbance was constructed to correlate absorbance with drug release levels. All experiments were conducted with triplicate polymer samples.

3.3.7 Cytotoxicity of drug released during 5 days of culture

For the preliminary dose-response study, COS-1 cells (10⁴ cells in 1 mL) were added to each well of a 12-well plate. The cells were allowed to adhere on the tissue cultures plates for 4 h at 37°C in a humidified 5% CO₂/95% air atmosphere, then an additional 2.5 mL of culture medium was added. Transwell inserts, each containing a sample of drug-loaded polymer (n=3 for each sample type), were then placed on top of the wells in the 12 well plates, so that each polymer piece was submerged in the culture medium. The cells were cultured for 5 days without a change of medium. During this time period, the drug incorporated into the polymer was released into the culture medium.
After 5 days of culture, the cell accumulation in each culture dish was assessed by a modification of a previously described procedure. Briefly, culture medium was removed and the cell layer was gently washed 2 times with 2 mL of phosphate-buffered saline (PBS). Cells were fixed for 1 hr in 0.5 mL of neutral buffered formalin, then stained for 1 hour with 0.5 mL of 1% toluidine blue in neutral buffered formalin. The dye solution was removed, the cell layer was washed 4 times with 2 mL of distilled water, and the plate was allowed to air-dry overnight at room temperature. All plates were scanned to make a photographic record, and the incorporated dye was subsequently dissolved by adding 0.5 ml 2 % SDS to each well and rocking the plate for 1 h at 25°C. Aliquots (3x100 µl) of the dissolved dye were read at 650 nm with a 96-well plate reader (VersaMax, Sunnyvale, CA).

3.3.8 Cytotoxicity of drug released during longer, timed incubation with cell culture medium

Polymer samples with incorporated drug were placed individually into tubes containing 2 ml of DMEM without serum or other supplements and incubated at 37°C in a humidified atmosphere of 5%CO₂/95% air. The entire medium sample was collected at a given time period interval and fresh medium (2 mL) was added to the polymer in the tube for the next incubation period. After incubation, the medium samples were stored in at 4°C refrigerator until cytotoxicity testing. For the zero time incubation point, each polymer was briefly dipped into 2 mL of culture medium and transferred immediately into another tube for subsequent incubation. The sample collection intervals were every 2-3 days; incubation time periods were shorter in the early days and longer after 20-25
days. For early dose-response experiments with films surface-loaded with MMC, the total incubation time was 8 days (192 h). For double-layered films, the total time that each polymer sample was incubated sequentially with culture medium was usually 34 days. After ~20 days of incubation, the culture medium became acidic due to breakdown of the PLGA matrix. A small aliquot of sodium hydroxide was added to these samples to adjust the pH to 7.4 before cytotoxicity testing. When such neutralization was necessary, a similar polymer sample with no incorporated drug was used as a control to insure that any toxicity observed was due to the drug and not to changes in osmolarity because of the neutralization process.

All samples were tested after the entire 34 day incubation period had been completed. The 2mL aliquot of DMEM that had been incubated with the polymer was mixed with 0.5 ml of culture medium supplemented with 50% FBS, 20mM glutamine, 5mM sodium pyruvate, 500IU penicillin, 500µg/mL streptomycin and 125µg/mL amphotericin B such that the final 2.5 mL sample mixture had the same composition as complete culture medium. COS-1 cells were plated into 12 well culture plates at 1x10^4 cells per well by adding 1 mL of cell suspension to each well. The cells were allowed to attach for 4 hours, as described above, then the 2.5 mL of DMEM that had been incubated with the polymer sample was added to each well. The final volume of culture medium in each well was 3.5 mL. The cells were incubated for 5 days with no medium change and cell accumulation was assessed as described above.
3.4 Results and Discussion

3.4.1 Coating of glaucoma device with breath figure PLGA films

In our earlier research, we used hydrogels loaded with MMC to inhibit fibroblast proliferation both in cell culture and in a GDD rabbit model\textsuperscript{55, 56}. While these hydrogels were effective and reasonably biocompatible, they are not biodegradable and may compromise long term implantation of the Ahmed valve. With a biodegradable material such as PLGA, the implanted surface seen after completion of wound healing is simply the inert silicone of the original Ahmed valve, once the PLGA film has completely degraded over a period of 30-60 days\textsuperscript{58, 59}. The sandwich design developed here to deliver MMC and 5-FU is simple and effective, and the coatings have the potential to deliver the drug effectively. The PLGA-coated Ahmed valve can be placed in the subconjunctival space and the release of drug in the area surrounding the end plate is triggered by the aqueous humor drained out of the anterior eye by the valve. The use of antimetabolites (MMC and 5-FU) has been shown attenuate the initial inflammatory reaction after trabeculectomies\textsuperscript{60} and such reduction in inflammation during the early phase of wound healing will lead to decreased fibrosis later in the wound healing process (for reviews, see 50, 61, 62).

Figures 11A and 11B illustrate the smooth surface of the silicone substrate over which the porous PLGA breath figure is spin coated. The PLGA film coating shown in Figures 11C and 11D illustrate the highly porous honeycomb structure of the breath figure.
Figure 11. Scanning Electron Microscopy Characterization of bare silicone and PLGA film coated silicone 
(A) and (B) shows the low and high magnification images of an uncoated medical grade silicone surface. 
(C) and (D) represents the low and high magnification images of silicone coated with porous PLGA film on the surface. The pores are created by evaporating a PLGA solution in the presence of moist air.

3.4.2 Loading and release of MMC from the surface of the breath figure PLGA films

The patterned microporous structure of the PLGA film provides a surface with the ability to bind and subsequently release pharmaceuticals, and preliminary studies were performed to evaluate these films for their ability to serve as reservoirs for the release of MMC. Figure 12A illustrates schematically the placement of MMC on surface
layers of the PLGA breath figure, while Figure 12B is an electron micrograph of the final breath figure structure, onto which MMC was loaded. This micrograph demonstrates that the surface PLGA layer retained its microporous structure even after MMC was loaded in a solvent mixture containing 5:1 methylene chloride /tetrahydrofuran. Because MMC can be inactivated under a wide range of conditions \textsuperscript{63}, these MMC-loaded discs were also tested for cytotoxicity. Silicone discs coated with PLGA alone or surface-loaded with varying concentrations of MMC (0.25, 1.0 and 5 µg) were tested for their ability to inhibit COS-1 proliferation during a 5-day culture period. The photograph in Figure 12C shows cell accumulation in a 12-well plate and visually demonstrates that MMC is released from the polymer films and inhibits cell proliferation. In the absence of MMC, the control polymer (labeled C) permits growth of COS-1 cells to a confluent monolayer. Cells cultured in the presence of the control polymer showed the same degree of confluence as cells grown without a polymer sample. This suggests that the PLGA was biocompatible and nontoxic to COS-1 cells during the 5 day incubation period. As the concentration of MMC on the polymer films increased, cell accumulation in the plate decreased. These data are quantified in Figure 12D, which demonstrate that MMC inhibits cell proliferation in a dose-dependent manner. The observation that 1-5 µg MMC was sufficient to significantly inhibit cell growth to 40% or less of that in control cultures was of importance in designing the drug loading of the later PLGA composite films.
Figure 12. Dose response of Mitomycin C and its in-vitro release from the surface of single layer porous PLGA film

**A)** Schematic illustration of the drug incorporation on the surface of breath figure PLGA film.

**B)** SEM image showing the porosity of the film after the drug incorporation.

**C)** Photograph of 12-well cell culture plate used for cytotoxicity testing after fixation and toluidine blue staining of COS-1 cells. The plate shows the toxicity of control PLGA films (C, no drug) and toxicity of PLGA films loaded with varying concentrations of MMC (0.25, 1 and 5 µg MMC)

**D)** Dose-response of COS-1 cell accumulation in response to PLGA films with different drug loadings. After solubilization of the cell-bound dye (toluidine blue), the color was a measure of the number of viable cells (quantified as absorbance at 650 nm). Ttests were used to determine if the samples with MMC were significantly different from the control; *, p ≤ 0.05, **, p ≤ 1 x 10^{-8}.

**E)** shows the *in vitro* release profile for films with 1 µg and 5 µg MMC loadings.

**F)** shows the corresponding cytotoxicity of drug released over an 8-day period.

Three individual polymer samples were tested for the data shown in Panels C-F and the results are expressed as the mean ± SD.
Figure 12
The experiments shown in Fig. 12C and 12D above do not provide any information about the kinetics of drug release, since they integrate the effect of MMC on growing cells over a 5-day culture period. The time-dependent release of MMC from these PLGA films into PBS is shown in Figure 12E for films loaded with 1 and 5 µg MMC, the concentrations most active in inhibiting cell proliferation. Clearly, most of the MMC was released very early after immersion into the PBS; this burst of drug release can be attributed to rapid dissolution of surface-bound drug. Based on absorbance at 364nm, which is characteristic of the active drug \(^{63}\), it appears that only 20-40% of active drug is released from the film. This was not unexpected, based on the reported sensitivity of MMC to extremes of pH, light and temperature \(^{63}\). The cytotoxicity of MMC released during timed incubation with cell culture medium provided release data similar to that for the \textit{in vitro} release into PBS, as shown in Figure 12F. In these experiments, the drug released in culture medium at each indicated time interval was individually tested for cell toxicity. These experiments also showed a very rapid release of anti-proliferative activity from the PLGA surface, with most of the active drug release within 1 day. The decrease in cytotoxicity over subsequent time intervals indicated that the drug was virtually depleted after the initial burst. Remnant levels of the drug adsorbed to the polymer were responsible for the cytotoxicity effects observed after the first 1-2 days. As was observed in the \textit{in vitro} release experiments, the IC\(_{50}\) for the PLGA-bound drug was \(~\)10 fold lower than that calculated for soluble MMC added directly to COS-1 cells.

Although the data in Figure 12 demonstrated that the anti-proliferative effect of MMC could be delivered from surface-loaded PLGA films, the drug release was too rapid to be effective for a glaucoma drainage device, since the fibroblasts that are
responsible for the excess fibrosis around the GDD do no migrate into the surgical site until later in the wound healing process\textsuperscript{50}. Attempts to delay the release of MMC by incorporating it directly into the PLGA matrix were unsuccessful because the MMC was rapidly degraded under these conditions and the degradation products were not cytotoxic. Experiments were therefore initiated to formulate 5-fluorouracil (5-FU), a stable, water-soluble antifibrotic agent commonly used in ophthalmology\textsuperscript{60, 64, 65}, into slow release PLGA breath figure films.

In order to achieve prolonged cell growth inhibition, we fabricated double-layered breath figure PLGA films, containing 5-FU as the major anti-fibrotic agent. Two different formulations systems were developed to achieve a continuous release of antifibrotic agent(s) over a period of ~1-30 days. Formulation I is illustrated in Figure 13A. The scheme shows the two layers of breath figure films fabricated one above the other. In formulation I, 5-FU was physically dispersed mainly into the bottom layer of a PLGA film and then sealed with another layer of thin film with no drug. SEM characterization (Figure 13 A1) reveals the porous structure in which the presence of 5-FU can be clearly observed (shown in circles). The representative electron micrograph corresponds to a film containing 0.4 mg 5-FU, before sealing the structure with another thin film. A second breath figure structure was created while coating the second layer (as shown in Figure 13 A2). To encapsulate the entire drug into the film, the bottom layer was spun at low speed, which resulted in a relatively thick drug-loaded bottom layer (~ 200 microns). The second layer was spin coated at higher speed to produce a very thin film (~ 20 microns). The creation of this second layer may disrupt to some extent the structure of the bottom layer, because the solvent used in the fabrication of the second layer could penetrate to
the lower layer and modify its structure. The shallower surface morphology of the second layer in the schematics of Figure 13A and 13B reflects this possibility. Formulation II is shown schematically in Figure 13B, with the difference being the surface coating of MMC. Like formulation I, these films were composed of a bottom layer containing 5-FU followed by a sealing layer. After the sealing, 1 µg of MMC was loaded on the surface while retaining the breath figure structure.
**Figure 13.** Schematic illustration of 5-FU incorporation into the double-layered breath figure PLGA films with and without MMC.

**(A)** (Formulation I) shows the 5-FU loading into the bottom PLGA layer and the sealed breath figure PLGA film at the top (with no added MMC drug). Panel A1 and A2 show the corresponding SEM images of the drug-loaded bottom layer (before sealing) and the top sealed film.

**(B)** (Formulation II) represents the 5-FU loadings into the bottom PLGA layer and 1 µg MMC loading on the top sealed layer.
3.4.3 In vitro release and degradation characteristics of 5-FU-loaded films (Formulation I)

The in vitro release profiles of double-layered PLGA films containing 5-FU are shown in Figure 14. Because of differences in scale, release kinetics for films containing 0.1 and 0.4 mg of 5-FU are shown in Figure 14A, while the data for films containing 2 mg 5-FU are shown in Figure 14B. As expected, the release pattern showed no burst effect in the first 24 hours of incubation, indicating that the sealing procedure was fully effective. The sealing delayed the initial release of 5-FU for 3-5 days. Subsequently, there was a sharp increase in drug release that continued for 10 days. Following this phase, the release entered a shorter lag period that lasted 2-3 days. The initial delay in the drug release provided additional time for the degradation of the bottom layer of PLGA film containing 5-FU. The lag phase is followed by a continuous release of drug for 21 days. Virtually identical absorbance data was obtained for Formulation II, because the small amounts of MMC surface-loaded onto these films was spectroscopically silent under the conditions used for these release experiments.

In previous studies, when drug moieties were incorporated into the single layered PLGA film (with no sealing), the drug release from the PLGA followed a tri-phasic profile. Immediate dissolution of surface bound drug resulted in a burst of drug release within 24 h. This was followed by an extensive lag phase during which the polymer was degraded with minor drug release. Once the polymer had been substantially degraded, the drug diffused out continuously with minor obstructions. In the formulations presented here, it appeared that the top sealing layer modified release profiles to prevent the initial
burst release. As the bottom layer drug-containing became exposed, the release that accompanied PLGA degradation occurred without a significant lag phase.

Figure 14. In vitro release characteristics of 5-FU from the double layered breath figure PLGA films (Formulation I) (A) shows the drug release from films containing 0.1 mg and 0.4 mg of 5-FU and (B) shows the release of 2 mg 5-FU. For all the drug concentrations, the delayed release observed is due to the top PLGA sealing layer.
3.4.4 Cytotoxicity of drug loaded double-layered breath figure PLGA films

3.4.4.1 Formulation I

The sensitivity of COS-1 cells to drug released from the double-layered PLGA films fabricated using formulation I is shown in Figure 15. The cytotoxicity of samples with 3 different levels of drug loading (0.1 mg, 0.4 mg and 2 mg 5-FU) was assessed. The cytotoxicity of drug released at different periods of incubation was quantified by culturing cells for 5 days, fixing/staining the cell monolayers with toluidine blue and measuring absorbance, as described in Methods. The data was normalized to obtain the percent of cells killed as compared to a PLGA film without drug. Figure 15A shows the results from the analysis of triplicate PLGA films containing 0.1 mg 5-FU. The graph shows variations in the toxicity among the three samples tested at early time points in the experiment. This heterogeneity was due to drug loading, film thickness and morphology. In addition, this cell-based cytotoxicity assay has a relatively narrow linear range and low level of drug incorporated into these samples enhanced our ability to measure small differences in cytotoxicity among the triplicate samples. At longer time points in the experiment (samples from >15 days of incubation with culture medium), the triplicate samples demonstrated much less variation, with a killing efficiency of about 75%.

The triplicate samples that contained higher concentrations of 5-FU (0.4 and 2 mg) showed better reproducibility in the cytotoxicity assays, as shown in Figure 15B and 15C. In the samples loaded with 0.4 mg of 5-FU, maximum cytotoxicity was not attained until the samples had been incubated with culture medium for a total of 4-5 days. This delay in drug release was due to the sealing of the drug-bearing layer with a second thin PLGA film. In the polymer samples that contained 2 mg of 5-FU (Figure 15C), the high
Figure 15. In vitro cytotoxicity of 5-FU loaded double layered breath figure PLGA films from Formulation I.

(A), (B) and (C) show the cytotoxicity for double-layered films containing only 0.1, 0.4 and 2 mg 5-FU, respectively. For 0.1 and 0.4 mg 5-FU, inconsistent toxic effect was observed for initial 5 days which is due to the sealed top layer with no drug content.
quantity of drug in these polymer samples most likely exceeded the linear range of our cytotoxicity assay. The cytotoxic activity eluted from these samples remained high from day 1 and stayed constant until the PLGA layer was almost completely degraded (see Figure 17, below). The effect of thin film sealing could not be observed in the assay in the presence of such a high drug dose.

3.4.4.2 Formulation II

The ideal drug delivery system for a GDD would have the ability to deliver a relatively constant dosage of anti-fibrotic medication during the wound healing process. Previous studies by Alvarado and coworkers demonstrated that adjunctive use of MMC intraoperatively and 5-FU and postoperatively during Ahmed valve surgery resulted in a higher long-term success rate, a lower rate of the hypertensive phase, and a lower rate of complications. This study demonstrated convincingly that delivery of anti-metabolites during the wound healing process could increase the long-term success rate of Ahmed valve implantation. We therefore surface-loaded 1 µg MMC onto the top, sealing layer of our PLGA films and the cytotoxicity of drug eluted from formulations with three different loadings of 5-FU is shown in Figure 16. For films containing 0.1 mg 5-FU loading (Figure 16A), the cytotoxic effect of eluate from the PLGA films during the first 2-5 days became much less heterogeneous. This was due the burst release of surface-loaded MMC, which occurred before the later release of 5-FU. Similarly, in the case of 0.4 mg 5-FU, the effect of MMC is apparent, as shown in Figure 16B. The inhibition of cell proliferation is profound throughout the experiment in the presence of moderate loading of 5-FU.
Figure 16. In vitro cytotoxicity of 5-FU/double layered breath figure PLGA films from Formulation II. % of COS-1 cells killed are shown for a period of 34 days. (A), (B) and (C) shows the cytotoxicity for 0.1, 0.4 and 2 mg 5-FU films in the presence of 1 µg MMC added to the top layer of breath figure film. The figures show the effect of adding MMC to overcome the inconsistency in the toxicity for the initial 5 days.
It is clear that the initial toxicity effect (5 days) is due to the release of surface bound MMC and in the later stage, the release of 5-FU continues to control the cell proliferation. Thus, films prepared by this formulation have the ability to continuously deliver anti-fibrotic medication for up to 28 days.

3.4.5 Degradation pattern of the 5-FU loaded double-layered PLGA films (Formulation I)

Figure 17 illustrates the morphological analysis of the degradation of the double-layered PLGA film over a period of 28 days. The film contains 0.4 mg 5-FU in the bottom layer with a sealing top breath figure PLGA layer with no loaded drug. Figure 17A and 17B show the surface and cross-sectional view of the degradation, respectively. Within 7 days, the deterioration of irregular breath figure pores was clearly visible. From the side view (Fig. 17B, day 7), the bottom layer had very little porous structure either in the bulk phase or in the thick dense layer of the film. With time (after 14 days) the film began to degrade and become permeable to aqueous medium. This generated irregular pores (pinprick pores) all over the film, which were not part of the original breath figure structure. The surface of the film also became slightly wrinkled due to the larger ingress of water, which can be observed from the side of view of day 14 degradation. After 28 days of incubation, the dense bottom layer was observed to be heavily swollen and wrinkled. We also observed that the polymer film stayed intact on the silicone substrate throughout the degradation process.
Figure 17 shows the change in morphology of the 0.4 mg 5-FU loaded double-layered PLGA film incubated for a period of 28 days. (A) represents the surface morphology of the degradation of the film characterized by SEM. (B) shows the cross-sectional view of the degradation.
The use of PLGA as a drug delivery vehicle has been widespread due to its biocompatibility, non-immunogenic nature and biodegradability under the physiological conditions\textsuperscript{58, 59, 67}. This study demonstrates the novel use of both MMC and 5-FU, with a small dose of MMC to provide the initial burst release to prevent fibroblast growth in the critical period immediately after surgery. The slow release of the less potent 5-FU over longer time intervals may allow the wound healing process to progress without scarring and blockage of the drainage conduit from the Ahmed valve. The use of breath figure morphologies is a simple variant of spin coating thin films but provides more open pore structures that can facilitate both drug loading and release. Additionally the design of layered structures with PLGA films is eminently feasible, allowing multiple combinations of fast and slow release with multiple drug species and the use of drug-free layers as sealants to control initial burst levels. The results indicate that the two drugs used in this study act in tandem to inhibit fibroblast growth for a period of 3-4 weeks during which most wound healing occurs.
3.5 Conclusions

The concepts that have demonstrated here can be easily extended to additional design elements of coatings on ocular implants and the incorporation of additional drugs shown to be effective in reducing fibrosis and extending the efficacy of the Ahmed valve \(^{52,68}\). Further studies is required on long-term results after implantation of the developed PLGA based drug delivery drainage system in rabbit animal models, paying particular attention to any inflammatory response and to the reduction of fibrosis after long-term implantation.
Chapter 4 - Fabrication And In Vitro Characterization Of A Breath Figure Based Antimicrobial Coating For Biological Surgical Meshes

4.1 Summary

Due to the various limitations associated with synthetic meshes, the biologically derived prosthetics are being preferred for abdominal wall hernia repair. However, when surgeons encounter highly contaminated fields, the biologic mesh is susceptible to bacterial colonization which leads to the rapid disintegration of the implanted mesh. The breath figure based porous antimicrobial coatings bonded to the surface of commercial biological mesh is described here. Vancomycin loaded porous poly (lactic-co-glycolic acid) (PLGA) and the composite poly (ethylene glycol) (PEG)-PLGA films were assessed for scanning electron microscopy (SEM) characterization, in vitro release kinetics and degradation pattern. Furthermore, the efficacy of drug-loaded polymer coatings to inhibit Methicillin-resistant Staphylococcus aureus (MRSA) for prolonged duration was examined, by correlating with the in vitro release profile. For both PLGA and PEG/PLGA coated mesh samples, the released drug is highly effective in inhibiting MRSA growth in vitro for four weeks; as characterized by modified Kirby-Bauer assay. The addition of 10% hydrophilic poly (ethylene glycol) (PEG) into PLGA showed an increase in the release rate and the release was complete in three weeks with continuously diffusion of drug throughout the test period. Furthermore, the in vitro degradation of breath figure PLGA and PEG/PLGA coated mesh leads to gradual deterioration of pores and formation of larger pores over a period of four weeks.
The formation of porous structures can be exploited for tissue remodelling while antibiotic is released locally at the implant site. The proposed approach could be highly effective and commercially scalable in developing infection-resistant biological meshes that can be successfully used in clinical settings for abdominal wall reconstruction.

4.2 Introduction

Although abdominal wall hernia repairs are amongst the most common and successful surgical procedures performed, the prevention of infection following surgery is of utmost importance\(^69,70\). In such surgery, the use of a mesh, either derived from synthetic or biological sources, is the commonly accepted material used to strengthen the abdominal wall and reduce the risk of hernia recurrence\(^71,72\). Due to the persistence of complications associated with synthetic grafts (chronic inflammation, mesh extrusion, bowel adherence, infection etc), biologically derived meshes with tradenames such as Surgisis\(^\text{®}\) and AlloDerm\(^\text{®}\) have emerged as better alternative prosthetic devices\(^69,73-75\). Surgisis\(^\text{®}\) for example, is a commercially available xenogenic tissue graft derived from porcine small intestinal submucosa. The graft primarily consists of a decellularized collagen based extracellular matrix (ECM), which acts as a scaffold into which cells can infiltrate and proliferate to promote neovascularization. In general, the biologic mesh is considered to induce less inflammation and is more compatible with normal tissue\(^76\). However, recent clinical data indicate that biologic tissue grafts have the potential to become colonized by pathogens and have to be used with caution\(^75,76\). In particular, bacterial infections accompanying the inflammatory reaction have a significant impact on reducing the integrity of the biologic mesh\(^69,76\). Hence there is a significant need to protect biological tissue grafts from the possibility of bacterial infection.
In the prevention of infection, the conventional systemic delivery of antibiotics through intravenous or intramuscular routes has been proven ineffective in killing sessile bacteria, which are well protected at the implant surface because of the formation of biofilm. The amount of antibiotics required to kill bacteria in the biofilm is roughly 1000 times more than the dose necessary to kill the equivalent level of planktonic bacterial cells. To prevent such bacterial colonization, researchers have attempted to develop antibiotic coatings for implants to deliver the antibiotic locally and effectively. To incorporate the antibiotics, various biodegradable polymers have been used as drug carriers, of which, the FDA approved polymer poly (lactide-co-glycolide) or PLGA is the most widely used. In addition to the controlled release of drug, PLGA based devices degrade naturally by hydrolytic process without leaving any toxic remnants. The biodegradable nature of the device eliminates the possibility of secondary surgery which is a normal practice to remove the infected implant.

In this work, we describe a simple and economically viable thin film PLGA based antimicrobial coating on the Surgisis® mesh, where a porous structure is easily fabricated using methodologies of generating breath figure structures. Vancomycin was chosen as an antibiotic agent and the drug was incorporated homogeneously into the polymer films. The drug loaded PLGA coating on the mesh was characterized by SEM to obtain morphological features. Moreover, drug incorporated PLGA and PEG/PLGA films were examined for release kinetics and in vitro degradation patterns. The modified Kirby-Bauer assay was utilized to evaluate the antibacterial effect of drug-loaded coated mesh for the incubation period of four weeks. Zone of inhibition was measured and correlated with the in vitro release profile. Gram positive bacteria,
Methicillin-resistant *Staphylococcus aureus* (MRSA) was chosen as a testing organism, as it is one of the most common pathogens found in implant related infections.

### 4.3 Materials and Methods

#### 4.3.1 Materials

Surgisis® mesh (Biodesign Surgisis® Inguinal Hernia graft), an 8-ply biologic tissue graft material with the dimensions 15 cm x 10 cm was obtained from Cook Biotech Inc. (Bloomington, IN, USA). Poly (D,L-lactide-co-glycolide) (PLGA 50:50) and poly (ethylene glycol) (PEG) polymers were used as drug carriers. PLGA 50:50 is a well-studied bioresorbable polymer which comprises 50% molar ratio of lactide and glycolide. The PLGA polymer (Resomer RG 506, Mw = 96000) was purchased from Boehringer Ingelheim Chemicals Inc., USA.

![Chemical structures of the Vancomycin used in the study.](image)

**Figure 18.** Chemical structures of the Vancomycin used in the study.
A low molecular weight hydrophilic polymer, poly (ethylene glycol) (PEG) (average $M_w=3350$ g/mol) was purchased from Sigma Aldrich Chemicals (St Louis, MO, USA). Vancomycin hydrochloride (Sigma Aldrich V-2002) (Figure 18) derived from *Streptomyces orientalis* was used as an antibiotic drug component. A model substrate, teflon of 0.2 mm thickness was obtained from Scientific Commodities Inc. (Lake Havasu City, AZ, USA). ACS grade dichloromethane was purchased from Fisher Scientific, USA. To prepare $0.1M$ phosphate-buffered saline solution (PBS), disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4$), potassium dihydrogen phosphate ($\text{KH}_2\text{PO}_4$), sodium chloride ($\text{NaCl}$), and potassium chloride ($\text{KCl}$) were all obtained from Sigma Aldrich Chemicals. For *in vitro* Kirby-Bauer analysis, gram positive bacterial strain, Methicillin-resistant *Staphylococcus aureus* (MRSA) was obtained from American Type Culture Collection (ATCC # 43300). All other chemicals were used as received without further purification.

4.3.2 Fabrication of breath figure PLGA-drug coating on Surgisis® mesh

Two different polymer-drug formulations, namely, PLGA and PEG/PLGA composite films were developed in this study and breath figure technique was utilized to create porosity in the polymer films. Prior to fabrication of antimicrobial coating on the surface of the Surgisis® mesh, the mesh was precisely cut into 8 mm diameter circular disks using a Harris Uni-Core™ sample cutter (Ted Pella, Inc., CA, USA). A spin coater (Laurell Technologies Corporation, PA, USA) was utilized to coat the surface of the mesh with a thin porous PLGA film containing Vancomycin. The PLGA polymer was dissolved in dichloromethane at a concentration of 15% (w/v). For each sample of the mesh coating, the theoretically calculated loading of vancomycin into the PLGA solution was approximately 37 mg drug per g of total polymer in a dry state. Immediately
following the dissolution of PLGA, a desired amount of vancomycin was dispersed into the PLGA solution and vortex-mixed for at least 5 minutes. The resulting PLGA-drug solution was observed to be homogeneous and appeared milky in color. To cast a thin film, the desired volume of polymer-drug solution was pipetted onto the mesh placed on the spin coater turn-table. Then, the mounting table was allowed to rotate to spread the polymer-drug solution uniformly over the mesh at a relatively low spin rate of 200 rpm for 10 minutes. The low spin rate prevents the solution from being spilled off the mesh, while accelerating the evaporation of solvent. During the spin coating process, the chamber was maintained at constant humidity (Relative humidity ~ 70%) to produce porous morphologies within the PLGA film. All mesh samples coated with the polymer films were dried for at least two days at room temperature prior to further analysis.

In another formulation, a small amount of the hydrophilic polymer, poly (ethylene glycol) (PEG) was blended at a weight ratio of 1:9 to PLGA to produce a composite film. A fabrication procedure similar to the PLGA coating procedure was followed. The thickness of all coated meshes was measured using a micrometer (Fowler) with a resolution of 0.001 inch.

4.3.3 Characterization of polymer coated mesh and in vitro antibiotic release

4.3.3.1 Scanning Electron Microscopy. We used room temperature field emission scanning electron microscopy (Hitachi S-4800 SEM) to characterize the morphologies of the uncoated mesh and drug-polymer film coated mesh. The samples were carefully cut and mounted onto the metal stub with a double sided carbon tape. The samples were then coated with a thin film of gold through sputtering (2 kV, 20 mA, 90 secs -Polaron sputter
coater). The surface and cross-sectional view of samples were imaged at an accelerating voltage of 3 kV.

4.3.3.2 *In vitro drug release and its degradation analysis.* To characterize the *in vitro* release kinetics, vancomycin loaded PLGA and PEG/PLGA films were immersed in 0.1M PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$ and 2 mM KH$_2$PO$_4$). For the release study, the antibiotic films were coated on teflon. The films coated on teflon disks (8 mm diameter) were placed in glass vials containing 1 mL of PBS (pH 7.4) and incubated at 37 °C. The vials were stirred using a horizontal shaker bath (Lab-line® Orbital shaking water bath) at a constant rate. At pre-determined time intervals, 0.650 mL of release media was removed, centrifuged and its UV absorbance measured (Shimadzu UV-Vis spectrophotometer, 1700 series) at 280 nm. In order to maintain perfect sink conditions, the glass vials were regularly replenished with 0.650 mL of fresh PBS solution. The release study was conducted for a period of 28 days. The release experiments were carried out in triplicate. The amount of drug released into the medium was calculated from a linear calibration constructed by measuring the absorbance from known concentrations of vancomycin dissolved in PBS (10 µg/mL - 100 µg/mL). The calibration plot has a regression coefficient of 0.9977. For *in vitro* degradation studies, the mesh samples coated with PLGA and PEG/PLGA films were immersed in 5 mL PBS solution and incubated at 37 °C. At weekly intervals of 7, 14, 21 and 28 days, the samples were withdrawn and rinsed carefully with distilled water before drying at room temperature. For all dried samples, the change in morphology was characterized by room temperature SEM after coating with a layer of gold.
4.3.4 Disk diffusion antibiotic-sensitivity analysis

The ability of the polymer coated mesh to inhibit the growth of the bacterial strain was examined by an *in vitro* Kirby-Bauer disk diffusion assay. The assay determines the susceptibility of a test microorganism to specific antibiotics, based on the zone of inhibition (ZOI). The test organism used in this study was gram-positive bacteria, Methicillin-resistant *Staphylococcus aureus* (MRSA). Using sterile forceps, surgical mesh samples (8 mm disks) coated with PLGA and PEG/PLGA films containing vancomycin were placed on Mueller-Hinton II agar plates. Prior to use, the plates were streaked for confluency with a single colony of MRSA isolated from a stock. The plates were incubated at 37 °C throughout the test period. After 24 h incubation, the diameter of the zone of inhibition (in mm) was measured and recorded. The samples were then transferred to freshly-streaked agar plates and incubated again for 24 hours. This process was repeated until no bacterial inhibition was noted. Controls with no vancomycin loading were maintained separately using the same procedure. Additionally, commercially purchased vancomycin susceptibility disks (30 µg) and an empty disk impregnated with 500 µg vancomycin solutions (equivalent to the drug content in polymer film coated mesh) were used for comparison. All the test samples were studied in triplicate and were UV sterilized for an hour, prior to Kirby-Bauer assay.
4.4 Results and Discussion

4.4.1 Surgical mesh characterization and fabrication of a thin polymer film incorporated with an antibiotic agent

In the spin coating process, the substrate can be coated to varying film thickness by altering process parameters such as spin turn-table acceleration, concentration and volume of the polymer solution. It is convenient to combine the spin coating with the breath figure process to fabricate a micro-porous polymer structure while forming a thin film. We have successfully developed antimicrobial loaded porous polymer films to coat a commercial hernia-repair mesh. In Figure 19, we have illustrated coating an 8-ply Surgisis® mesh with a thin porous drug-loaded polymer film. Figure 20A shows the photographic image of commercially purchased Surgisis® Inguinal hernia mesh. The mesh is uniformly aligned with holes for suturing purpose when the mesh is placed onto the back of the abdominal wall fascia.
Prior to coating with antimicrobial films, scanning electron microscopy (SEM) was used to examine the morphology of uncoated mesh. A closer observation using low resolution SEM (Figure 20B) reveals groove-like herringbone patterns on the surface with dimensions of approximately 600 µm x 150 µm (length x width) in size of each groove. The average thickness of the mesh substrates measured by micrometer is 175 µm. In Figure 21, we have shown SEM images of the spin coated and drug loaded breath figure PLGA film on the surface of the mesh. In figures 21A-21C, the highly porous nature of the PLGA film coatings are shown with increasing magnification. As seen from Figures 21A and 21B, the porous PLGA film covers the surface pattern of the mesh with the porous features seen over the groove patterns. Although the pores obtained are not
entirely uniform, they are prevalent throughout the surface. The pore dimension is in the range of 2 - 4 microns in size. Upon drying, we observed that the thin PLGA film adheres firmly to the mesh and is not easily peeled off from the mesh. The average thickness of the spin coated PLGA film measured by a micrometer is 20 µm.

Figure 20. (A) Photograph of Surgisis® mesh (15 cmx10cm). (B) Scanning Electron Micrograph (SEM) showing the herringbone groove pattern on the surface.
**Figure 21.** SEM images of the Surgisis\textsuperscript{®} mesh coated with the breath figure PLGA film. 
(\textbf{A-C}) shows the surface morphology of breath figure film at progressive magnifications illustrating the porous surface structure. 
(\textbf{D}) Illustrates the cross-sectional view of PLGA film coated mesh. The interconnected porous structure is clearly seen in the bulk phase of the film.
Figure 21D illustrates the cross-sectional analysis and reveals both surface and bulk features of the PLGA coated mesh indicating the surface and multi-layered porous structure of breath figures\textsuperscript{58}.

4.4.2 Vancomycin release from breath figure PLGA and PEG/PLGA films

In our earlier work, we have shown that breath figure films loaded with a hydrophilic drug have increased release rate in comparison with nonporous films of the same material\textsuperscript{58}. Here, we show release kinetics of vancomycin from porous breath figure films fabricated with PLGA or with PEG/PLGA. We have deliberately chosen to use Teflon as the substrate for the breath figure coating rather than the mesh since we find that slow degradation of the tissue based mesh and release of compounds in the mesh leads to interference in the absorption spectra of vancomycin in particular at the optimal wavelength of 280 nm. Thus, to avoid the complexity involved in quantification of drug release due to the spectral interference, we have used Teflon as the substrate maintaining the identical spin coating conditions used to coat the mesh.

The release experiment from the breath figure PLGA and PEG/PLGA films was done at physiologic conditions (37°C, PBS-pH 7.4). Figure 22 illustrates the normalized release profile of breath figure PLGA and PEG/PLGA films, respectively. The PLGA film shows the typical three phase release profile of such biodegradable films\textsuperscript{17, 30, 59} that have been well studied in the literature. The three phases include an initial burst release, a diffusion controlled lag phase and a polymer degradation controlled phase\textsuperscript{43, 86, 87}. Although there is an initial burst release of about 20% of the drug over the first day, we note that the release takes place over a period of 18-20 days. In the case of the
PEG/PLGA film, the presence of 10 wt% PEG modulates the release profile of vancomycin with a minimal lag phase. The addition of a small amount of hydrophilic PEG increases the release rate by absorbing large amounts of water into the film. In comparison to the PLGA release, there is a nearly two-fold increase in release rate from the PEG/PLGA film in the initial time period of drug release. At day 9, approximately 72% of the drug was released from PEG/PLGA coating as compared to 40% from the PLGA coating. At this stage, the release profile follows an approximately constant release rate (zero order profile). Similar release profiles have been observed by Wang and coworkers who have reported the reduction of the lag phase upon incorporating PEG into PLGA films for the release of sirolimus. It is speculated that the dissolution of surface bound hydrophilic PEG disrupts the film integrity that results into the formation of larger channels and voids, thereby accelerating the degradation and increased release of drug from the composite PEG/PLGA film.
4.4.3 *In vitro degradation*

In order to correlate structure with the release kinetics, the morphological changes of vancomycin loaded polymer films coated on the surgical mesh were monitored throughout the release period of four weeks under physiological conditions (PBS with pH 7.4, $37^\circ$C). SEM imaging was used to observe the changes in surface features including porosity. The panels of Figure 23A represent the morphological changes of the PLGA film due to degradation. After 7 days of incubation, clear morphological changes (pore size $\sim 4$ µm) were observed with the deterioration of the original breath figure pores.
(pore size ~ 2 µm) of the drug-loaded PLGA film. The initial degradation is mainly due to the dissolution of surface bound drug particles into the release medium controlled by diffusion mechanism, which can be related to its release profile (Figure 22). As degradation proceeds to 14 days incubation, the film reveals the underlying porous network and the increase in the number density of pores formed on the surface. After 21 days, the formation of sub-micron pores increased substantially with the significant loss of degrading polymer. This can be seen from the formation of surface wrinkles with little swelling and the film no longer maintains the flat structure (shown by arrows). At the end of 28 days, the film shows no uniformity on the surface feature and much of the film has eroded away. During the course of degradation, we observed that the structural integrity of the film bonded to the mesh is maintained throughout the incubation period.

The panels of Figure 23B reveals the degradation of breath figure PEG/PLGA films containing vancomycin. In addition to the effect of vancomycin elution, the presence of PEG contributes significantly to the changes in morphology. The presence of large numbers of inter-connected porous networks can be seen upon 7 days of incubation. Essentially, the addition of small amount of PEG accelerates the degradation rate as we observe clear morphological change with increase in porosity throughout the surface. We also note that the amount of wrinkles and swelling (shown by 5-point star) is higher in the PEG/PLGA film in comparison to the PLGA film. After 21 days of degradation, the pore size of degraded film has increased to approximately 10 microns.
Figure 23. SEM micrographs showing degradation of breath figure PLGA and PEG/PLGA films coated on the surgical mesh for 28 days incubation. (A) Surface morphologies of PLGA film and (B) PEG/PLGA film. All scale bars denote 20 µm.
4.4.4 Disk diffusion antibiotic-sensitivity analysis

For all in vitro Kirby-Bauer disk diffusion assays, methicillin resistant staphylococcus aureus (MRSA) was chosen as a bacterial strain, as it is one of the most common pathogens associated with mesh related infections. The Kirby-Bauer assay was used to determine MRSA susceptibility to the slow release of vancomycin from porous polymer films coated mesh. First, control samples with two different drug loadings were tested, as shown in Figure 24. Figure 24A illustrates the assay results for a commercially obtained vancomycin susceptibility disk containing 30 µg of drug, where the zone of inhibition (ZOI) is plotted measured against the release time. The ZOI is obtained by the formation of ring-like clearance zone surrounding the disk (inset of figure 24A). The size of ZOI (measured in diameter) around the disk implies the proportionality to the susceptibility of MRSA to vancomycin. The diffusion of vancomycin lasts for only two days, with a minimum of 12 mm ZOI, which indicates MRSA is likely to respond to the antibiotic. In the figure 24B, ZOI is shown for the disk containing 500 µg of vancomycin (approximately equivalent to the loadings in polymer films).

Figure 24. Kirby-Bauer Analysis for control samples indicating the zone of inhibition (ZOI) data as a function of time.  
(A) Commercially available disc containing 30 µg vancomycin  
(B) Empty disc containing 0.5 mg vancomycin with no polymer coating  
The insets in Figure 24A and 24B are corresponding photographs of the clearance zone (after 24 hrs)  
(C) SEM of the control uncoated mesh (24 hrs). The mesh is completely filled with MRSA (no inherent antibiotic activity). The inset to the figure confirms the lack of a zone of inhibition.
Figure 24
The disk was prepared from a filter paper using a hole punch and impregnated with vancomycin solution (dissolved in sterile water). Higher loadings of drug results into burst release followed by a continuous decrease in concentration. In comparison to figure 24A, the increase in drug loadings to about 17 times may have extended the release for four days, but there is no significant difference in ZOI. The drug release shows initial burst followed by the continuous depletion of drug until four days. The inset to figure 24B shows the ZOI picture taken for day one release. For effective therapeutic potential, the release at high dosage locally at the implant site requires control in kinetics, which may otherwise trigger systemic toxicity. Therefore, the coating of the mesh surface with a thin polymer film incorporated with therapeutic agents renders controlled release of the drug in achieving desired antibacterial effect at the surgery site. In the case of surgical mesh with no polymer coating, we observed no ZOI surround the mesh after a day of
incubation (inset to Figure 24C). As characterized by SEM analysis (Figure 24C), MRSA is found to be adhered and populated well all over the surface of mesh. This suggests that the mesh alone does not induce antibiotic effect against MRSA.

**Figure 25.** Kirby-bauer analysis for breath figure PLGA coated mesh containing Vancomycin  
(A) Zone of inhibition (ZOI) data for 28 days of incubation  
(B) Pictures of ZOI taken at different time intervals  
(C) Control PLGA coated mesh with no vancomycin content
Figure 25
Figure 25A shows the effect of porous PLGA film coated mesh in inhibiting MRSA growth *in vitro*. The amount of vancomycin present in each sample of PLGA coated mesh examined is approximately 500±50µg. The drug released from the coated mesh is effective and maintains activity against MRSA for a period of 25 days. Throughout the test period, the ZOI is maintained for at least 11 mm diameter (Intermediate susceptibility). A 15-mm diameter ZOI, corresponding to the burst release is observed during 24 h incubation. Upon implantation of mesh, the initial bacterial adhesion is critical to prevent the implant-related infection. Within 24-48 hrs, the adhered bacteria become sessile and secrete exopolysaccharide to protect the colonies of bacteria and subsequently, the formation of biofilm. To prevent an initial adhesion of free-flowing bacteria, the burst release provides high dosage of vancomycin (several hours up to 1 day) at the implant surface. The burst release is followed by a lag phase that continued until 6 days, which can be correlated well with the release profile of PLGA film (Figure 22). Following the lag phase, the degradation of the polymer allows continuous release of drug for prolonged duration. During the degradation phase, the ZOI is again maintained above 10 mm diameter, as evident from the inhibition plot. The result of ZOI is comparable to the release profile of PLGA film, as previously described in Figure 22. In the Figure 25B, the photographs of PLGA coated mesh placed in the MRSA-streaked agar dish taken at different time intervals of the study (day 4, 9, 14 and 22) are shown. The inhibition zone size is nearly constant for all the time points described here. The control PLGA film coated mesh with no drug is shown in Figure 25C, which reveals the growth of MRSA surround the entire polymer film and the mesh. This
suggests that either PLGA or mesh show no inherent inhibition towards bacterial adhesion to the implant surface.

Figure 26. Kirby-bauer plot for breath figure drug loaded PEG/PLGA film coated mesh. The graph shows ZOI data with respect to incubation time period of 28 days.

Figure 26 shows the effect of drug release from the porous PEG/PLGA film in inhibiting MRSA stain. Similar to the observation from the porous PLGA film, the released drug from the porous PEG/PLGA film is active against MRSA for four weeks. ZOI is very much comparable to the results from the PLGA film. As it is seen from the plot, the extended burst release was obtained (upto 72 hrs). We assume that the rapid
disintegration of PEG from the PEG/PLGA film extended the burst effect for the first 3 days, followed by a lag phase. However, in the release kinetics, no lag phase is observed for the PEG/PLGA film (Figure 22) and shows discrepancy with respect to the Kirby-Bauer plot. This can be explained from the experimental conditions followed for both release study and Kirby-Bauer test. Typically, the release study requires aqueous medium stirring at a constant rate to prevent the formation of a stagnant layer. The elimination of stagnant layer is important to maintain concentration gradient in the polymer film, while the drug is diffused out from the bulk phase. However, in the case of Kirby-Bauer assay, the diffusion of drug occurs mainly by absorbing water into the disk from the solid wet agar. The release of drug from the disc placed on the agar medium is expected to be slower than the release from the PBS medium. Therefore, the diffusion rate of vancomycin in the agar plate is not as rapid as the extraction rate of the drug out of the disk. Moreover, the rate of diffusion of the vancomycin is highly dependent on the concentration, diffusion, molecular weight and solubility properties of the drug in agar medium\textsuperscript{89}. Based on the results observed from these \textit{in vitro} experiments, it is apparent that the porous antimicrobial films coating of Surgisis\textsuperscript{®} mesh renders sustained release of drug and highly effective against gram positive bacteria, MRSA.
4.5 Conclusions

In this study, we have described the application of breath figure PLGA and PEG/PLGA films in releasing Vancomycin from the commercially available Surgisis® mesh. The fabricated samples were successfully examined for in vitro release kinetics, degradation pattern and correlated the results with the Kirby-Bauer assay tested against MRSA. This novel design of antibiotic coated mesh released the vancomycin in a sustained manner and the released drug was active against killing MRSA for four weeks. The breath figure based thin polymer coating of medical devices offers a versatile approach and can be applied to a variety of medical implants (glaucoma devices, stents, catheters, sutures etc) for sustained drug-elution from the device surface. In addition to the drug release, the porous film is expected to render increased durability and mechanical properties of the mesh and also promote faster vascularization and tissue healing in the post-operative wound healing time period.
Chapter 5 - A Novel Three-dimensional PLGA Scaffold System Using The Breath Figure Method For In Vitro Evaluation Of Mammary Morphogenesis And Chemotherapy Response

5.1 Summary

This study describes the novel uses of biodegradable poly(lactic-co-glycolic acid) (PLGA) scaffolds to recapitulate three-dimensional (3D) mammary growth and function in vitro. An easily implemented water-templating breath-figure technique was used to generate scaffolds with honeycomb-structured pores that varied in sizes from 2-12 µm in spin coating, and 5-30 µm in dip coating procedures. Following a week-long culture, growth of MCF-7 cells on these scaffolds was characterized using phase contrast, scanning electron microscopy, histopathology and confocal microscopy. 3D PLGA scaffolds showed slower growth kinetics and higher epithelial differentiation in comparison to the 2D controls. Changes in lobulo-alveolar volume and surface expression of K18 and E-cadherin confirmed their acinar phenotype. Specifically, dip coated PLGA supported more robust 3D growth (1.88 times larger lobular-alveolar size) in comparison to the spin coated PLGA. Gene profile analysis (GATA3, EMA and INTEGβ4) indicated that the 3D scaffolds induced enhanced expression of mammary differentiation genes even in the absence of hormonal stimuli; and per expectation expression of mesenchymal gene (CALLA) was down-regulated.
Importantly, cells grown on 3D PLGA systems exhibited an enhanced resistance to doxorubicin treatment in comparison to 2D cultures \((p < 0.05)\). Breath-figure PLGA scaffolds show promise in mimicking \textit{in vivo} mammary functions and can potentially be used to screen chemotherapeutic drugs. The simplicity and ease of fabrication of these morphologies are especially appealing to the development of effective scaffold development in tissue engineering.

\section*{5.2 Introduction}

Postnatal mammary ductal development occurs during puberty through ramification of terminal end buds (TEB) to give rise to a ductal network consisting of luminal and myoepithelial cells. The luminal cells, as the name suggests, line the lumen of the duct, whereas the myoepithelial cells stay in direct contact with a laminin rich basement membrane. This bilayered epithelium stays embedded in a fatty stroma. Hormonal changes during puberty, pregnancy and lactation result in terminal differentiation of the ductal cells into milk producing acini \cite{90, 91}. The dynamic nature and complexity of mammary morphogenesis is a big challenge when it comes to recapitulating this process \textit{in vitro}. However, as most breast cancers arise in ducts from cyst-like structures that fail to differentiate into ductal and alveolar cells\cite{90, 92, 93}, it is paramount to develop \textit{in vitro} model systems that recapitulate branching morphogenesis and retain all the essential features of a typical breast tissue including the stiffness afforded by the extracellular matrix (ECM). These \textit{in vitro} models are particularly needed to understand how differentiation gone awry can result in breast cancer growth,
and to carry out meaningful screening of anticancer agents which are effective against breast cancer cells and recapitulate their in vivo efficacies.

Traditionally, cells cultured in two dimensional (2D) petri-dishes have been used for evaluating the cytotoxicity of anticancer agents prior to testing in animal models and preclinical trials. However, cells grown on 2D surfaces lack the physiologically relevant microenvironment and any resultant cues emanating from reciprocal signaling between the epithelium and the underlying mesenchyme (fatpad) as seen in the breast tissue \(^94, 95\). An emerging consensus therefore is that traditional 2D cell culture may not accurately mimic the three-dimensional (3D) environment in which cancer cells reside. Specifically, the unnatural 2D environment may produce inaccurate data regarding the predicted response of cancer cells to chemotherapeutics\(^96\). For instance, breast cancer cells grown in 3D environment show greater resistance to therapeutic agents, slower proliferation rate, different cell density, reduced sensitivity to apoptosis and distinct gene expression profiles in comparison to cells in 2D systems\(^96-99\). This is also true for ductal development where formation of lumen and ductal structure is distinctly influenced by its 3D surroundings\(^94, 100, 101\). Such discrepancies between 2D and 3D cultures and the complexities of available animal models have prompted researchers to devise alternative in vitro model systems that are convenient, reproducible and possess relevant multifunctional and dynamic microenvironment characteristics\(^102\). Therefore cultures which allow the growth of multi-cellular 3D structures and retain essential features of a typical breast tissue may be invaluable to drug development.

Whereas the perfect method for 3D cell structures may not have been determined, clearly a lot of effort is going into engineering inherently active biomaterial scaffold that
are reproducible, are commercially scalable and can produce the most in vivo-like structures possible. Additional focus has been to ensure that the biomaterial possesses biomechanical and biocompatible properties to support sufficient cell in-growth and differentiation to form living tissue-like structures. In addition, proper combination of signals delivered by biological growth factors and the ECM may be required for emulating in vitro a niche that can drive cells towards a defined phenotype.

Recently, three-dimensional cultures of mammary cells in collagen gels, commercial Matrigel, chitosan scaffolds and micro-patterned arrays have been used extensively to replicate in vivo growth with varying degree of success. However, the matrix stiffness, required to drive the commitment of mammary precursors to differentiated mammary architecture is lacking in these systems. Here, we chose to develop scaffolds based on FDA approved poly (lactic-co-glycolic acid) (PLGA50:50) polymer, which renders necessary stiffness and pliability to regulate numerous cell functions. For this purpose, a new breath figure technique has been utilized to create porous PLGA scaffolds with varied pore sizes. The topography with appropriate roughness and highly inter-connected multi-layered porous structure can facilitate cell adhesion, proliferation, differentiation and eventual tissue regeneration in a natural manner. Although, the honeycomb structured polymer scaffolds have been investigated for the growth of various cell lines, including, hepatocytes, neural stem/progenitor cells and endothelial cells, they have not been fully examined as 3D model system for any breast epithelial cell line including the most representative MCF-7 cells. Based on Tanaka’s reported findings on growth of human cancer cells on honeycomb films, our objective was to evaluate how the 3D environment of PLGA
based honeycomb scaffold system influences the growth kinetics, differentiation program and the chemotherapeutic response of MCF-7 cells as compared to their growth under 2D environment.

Here, we describe the synthesis of various breath figure PLGA porous scaffolds fabricated with spin-coating and dip-coating techniques. MCF-7 cells cultured using these scaffolds showed slower growth kinetics and more differentiated phenotype when compared with cells grown in 2D surfaces. We also demonstrate significant differences related to the effect of the chemotherapeutic drug doxorubicin on cell proliferation using MCF-7 cells growing in 3D and 2D cultures. Our findings indicate that this novel and engineered breath figure PLGA scaffold may be an effective and alternative biomaterial to develop drug screening protocols against breast cancer cells and recapitulate the branching morphogenesis seen in \textit{in-vivo} model systems of normal breast epithelial cells.

5.3 Materials and methods

5.3.1 Materials

Poly (D,L-lactide-co-glycolide) (PLGA 50:50) polymer (Resomer RG 506) was purchased from Boehringer Ingelheim Chemicals Inc. (Petersburg, VA, USA). The coating substrate, microscope cover glass of diameter 24 mm, was purchased from Fisher Scientific Inc. (Pittsburg, USA). Dichloromethane (organic solvent, ACS grade) was obtained from Fisher Scientific Inc. (Pittsburg, PA). All materials and chemicals were used as received without further purification.
MCF-7 cells were maintained in Minimum Essential Medium, Eagle with Earle’s balanced salt solution (MEM) (ATCC, Manassas, VA) supplemented with 0.05% Insulin (2 mg/mL), 1% Fungizone, 1% Penicillin-Streptomycin (P/S) solution and 10% Fetal bovine serum (FBS). The anticancer drug, doxorubicin hydrochloride was purchased from Sigma Aldrich. 0.25% Trypsin-EDTA (Invitrogen) and Dulbecco’s phosphate buffered saline (DPBS) (Cellgro, Mediatech Inc, Manassas, USA) were used as received. For all cell culture experiments, 70-80% confluent cells in a T-25 flask were used.

5.3.2 Synthesis of “Breath figure” PLGA scaffolds

Breath figure method and coating techniques were combined to fabricate porous thin PLGA scaffolds with various pore size ranges. Prior to coating, the cover glass was acid-treated and UV-sterilized for an hour.

5.3.2.1 Spin-coating procedure: A manual spin coater (WS-400-6NPP-LITE, Laurell Technologies Corporation, North Wales, PA) was used to fabricate a thin scaffold on the glass substrate in the presence of moist air (Figure 27). The spin coater was connected with a tube from which the humid air stream was passed into the coating chamber throughout the coating process. By bubbling air continuously through the distilled water, the humidity (~70% Relative humidity [RH]) was created and maintained. The PLGA polymer was first dissolved in dichloromethane at a concentration of 10% (w/v). Then, the polymer solution was dropped onto the glass substrate and immediately accelerated to 2500 rpm for 30-40 sec to spread the solution uniformly over the substrate. The solvent was evaporated out quickly and thereby, facilitating water droplets to condense from the humid air to create porous cavities into the polymer film. Macroscopically, the initial
transparent polymer solution turned opaque due to the emulsification of water droplets in the breath figure process. The samples were then dried for at least a day at room temperature (RT) before using them for cell culture experiment.

5.3.2.2 Dip-coating procedure: In the case of dip coating, the polymer solution (10% w/v in dichloromethane) was taken in a glass beaker (Figure 27). Using a tweezer, the glass substrate was dipped into the polymer solution. After allowing the substrate to coat with the polymer film for about 10-20 sec, the substrate was removed and transferred into the closed chamber maintained with constant humidity (~70% RH). Inside the chamber, the coated substrate was placed in a slanting position in a glass petri-dish. This step was followed by blowing humid air for 30 minutes to an hour. Samples were then dried at room temperature for at least a day. The film formed on the unexposed (bottom) side of the cover glass was peeled off and discarded once the polymer was sufficiently dried. In contrast to spin coating (< 10 µm pores), the evaporation of solvent is slow and steady during the dip coating procedure which results into increased condensation and penetration of water droplets into the polymer (> 10 µm pores).

5.3.2.3 Preparation of 2D substrate: To compare the results of cell culture on porous PLGA scaffolds, we used a microscope cover glass and non-porous PLGA films as control 2D samples. The non-porous PLGA was prepared similarly using the spin coating procedure under nitrogen atmosphere (< 10% RH). In order to promote cell adhesion, the glass cover slips were initially treated with 1N HCl solution for 30 minutes to an hour, rinsed thoroughly with distilled water and stored in absolute ethanol. Prior to using them for cell culture or coating with the polymer, they were either air dried or heated over a flame to evaporate off the ethanol.
Figure 27. Experimental set up illustrates the fabrication of breath figure PLGA and cell culture on these porous scaffolds in vitro. Two different pore sizes were obtained simply by altering the fabrication procedure. (A) The scheme illustrates the MCF-7 cells seeding onto the porous polymers (regardless of pore sizes). The samples were preconditioned with the cell culture medium overnight prior to cell seeding. After 7 days of cell culture, the formation of 3D tissue-like structure is depicted in the schematic diagram. (B) When the cells are seeded in 2D control cover glass (no polymer coating), the cells grow as monolayer on the surface.
All PLGA samples (both 2D films and 3D scaffolds) and the cover glass were UV-sterilized for an hour and conditioned with the cell culture medium (MEM) overnight at 37°C in an incubator before plating of the MCF-7 cells.

5.3.3 Characterization of breath figure PLGA scaffolds

Surface and cross-sectional morphologies of PLGA samples (spin-coated, dip-coated and non-porous films) were characterized by scanning electron microscopy (SEM; Hitachi Field Emission S-4800) operated at an accelerating voltage of 3 kV. The samples were subjected to sputtering (Polaron SEM sputter) with a thin gold layer (15 mA, 2.4 kV, 90 sec) prior to SEM imaging. To obtain the side view, a cross-sectional cut of the film was gold coated as well. Imaging software (Image-pro Plus version 5.0) was used to measure average pore size and pore size distributions. A minimum of five SEM images were used in analysis with 300 pore dimensions to obtain statistics on pore size distributions.

5.3.4 Cell culture on breath figure PLGA scaffolds and 2D controls

Spin coated or dip coated porous 3D PLGA scaffolds, and the cover glass with non-porous 2D PLGA films were placed in six-well tissue culture plates for preconditioning. The schematic of cell seeding and growth is depicted in (Figure 27). MCF-7 cells were trypsinized and counted using a hemocytometer. For all cultures, the seeding density was 5 x 10⁴ cells per well unless indicated otherwise. The cells were allowed to attach to the substrates overnight. Once attached, the substrates were transferred to new six-well plates. This time point was defined as day zero for all the experimental procedure. The samples were transferred to new plates, and any cells
directly attached to the bottom of the wells were counted and subtracted from the total number of cells seeded, in order to obtain the number of cells on the transferred scaffolds. All cell cultures were incubated at 37 °C, 5% CO₂ in a 100% humidified incubator during the 7 – 11 day period of study. The cells were replenished every two days with fresh MEM culture medium. Throughout the experiment, cell growth was monitored with optical microscopy (Meiji, Precision Instruments LLC). After 7 -11 days, cells growing on the scaffolds or coverslip were fixed using 4% buffered formalin solution (16% paraformaldehyde, Electron Microscopy Sciences, PA) for 20 minutes at 4° C prior to further analysis.

5.3.5 Microscopic analysis of cell morphology and growth on breath figure 3D PLGA scaffolds and 2D controls

Following formalin fixation, the morphology of cells seeded in both 2D and 3D samples was assessed by toluidine blue staining, SEM and confocal laser scanning microscope analysis. For visualizing the morphological changes, fixed MCF-7 cells grown on various substrates were stained with 0.5% toluidine blue solution (Polyscientific R&D Corp., NY, USA) for 10-15 minutes. Any excess stain was removed by rinsing the samples carefully with distilled water, following which they were analyzed using EVOS phase contrast microscopy (AMG, Bothell, WA). Multiple images were captured to assess morphological development of MCF-7 cells in porous 3D PLGA scaffolds (spin coated and dip coated) and compared with the controls (cover glass and non-porous 2D PLGA).
To further confirm any changes in morphological features, we carried out topographical analysis of our specimens using SEM (Hitachi S-4800 Field). For these studies, the fixed specimens were rinsed with sterile water and air dried at room temperature for at least a day, sputter coated with a gold layer before SEM analysis.

5.3.6 Confocal Analysis: After 7-11 days of culture, the scaffolds were transferred to a new six well dish and washed with DPBS to get rid of serum and other particulate matter. The scaffolds were then fixed in freshly prepared 4% buffered formalin for 30 minutes to an hour at RT, washed with DPBS three times and stored at 4°C overnight or until use. The 3D morphology of dip coated PLGA scaffolds and 2D cover glass samples were obtained by imaging using the immunofluorescent technique of labeling the fixed cells (on various samples) with primary antibody followed by detection with a fluorescently tagged secondary antibody. After formalin fixation and rinsing with 1x DPBS, the samples were blocked for an hour with 10% normal goat serum (GIBCO, Invitrogen) prepared in PBS-T wash buffer (1 X PBST contains 3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, 0.05% Tween 20, pH 7.4.). After blocking, the samples were stained with primary antibody and incubated overnight at 4°C. Keratin 18 (1:1000 µL dilution) (Thermo Scientific Inc) and E-cadherin (32A8 Mouse mAb, New England BioLabs, kindly shared by Dr. Srikanta Dash, Tulane University) were chosen as the primary antibody and were diluted with blocking buffer containing Tween-20. After primary antibody treatment, specimens were washed three times with PBST for 5 minutes in each cycle to remove unbound primary antibodies. Following which samples were treated with fluorochrome conjugated secondary antibody (Alexafluor 488; goat antimouse IgG, Invitrogen) at 1: 250) dilution and incubated in the dark for 1-1.5 h at
room temperature. Prior to nuclear staining, the samples were washed again with PBST buffer to remove any non-specific secondary antibody binding. Finally, all the specimens were counter-stained using DAPI (Invitrogen) dye. DAPI stains the cell nuclei and fluoresces blue color. The labeled specimens were then mounted carefully on superfrost plus microscope slides (Fisher scientific). Images were acquired with a 20x objective lens using a Leica TCS SP2 confocal laser scanning microscope system (Leica Microsystems, Germany). Immunostaining in the acinar structures was examined using confocal microscopy with a blue (405 nm) and green (488 nm) excitation filter sets. In control specimens, the primary antibody was omitted and the samples were just treated with blocking buffer and incubated over night at 4°C, followed by secondary antibody incubation and DAPI staining similar to the experimental samples.

We also examined the effect of addition of differentiating agents on the growth of MCF-7 cells for both dip coated porous PLGA and 2D cover glass. To induce differentiation, the MEM medium was supplemented with differentiating agents, Dexamethasone (1 µM) (Sigma Aldrich), Hydrocortisone (1 µg/mL) (Sigma Aldrich), Insulin (5 µg/mL) and Prolactin (10 ng/mL) (Sigma Aldrich). The differentiating agents were fed after the cells were allowed to attach and grow for four days. The experiment was continued for the following seven days. For confocal analysis, similar staining procedures were followed for both differentiated and undifferentiated cells.

5.3.6.1 Analysis of ductal outgrowth morphology

Individual cysts/acinar units at different regions of the scaffold were imaged in whole mounts of the day 7 culture preparations by confocal microscopy with a 20x objective (Leica TCS SP2). Growth on 2D coverslips was also examined using the same
procedures. Serial optical sections were taken sequentially from the surface through the whole thickness of the acinar units in the scaffold. These optical sections were stacked to give maximum projected image of all ductal and lobular units within this area using Leica software. Only growth cones that had a distinctive appearance as branched units with lumen containing lobular units were chosen for analyses. Thickness of growth in projected confocal images was also measured through z stacks imaging. Numerical results were analyzed using the Mann-Whitney Rank Sum Test.

5.3.7 Proliferation of MCF-7 cells on 3D PLGA and 2D samples

To study the effect of porosity on cell proliferation, a growth kinetics study using trypan blue assay (0.4% solution, Sigma Aldrich) was performed over a period of 8 days. Briefly, $2.5 \times 10^4$ MCF-7 cells/well were cultured on preconditioned porous 3D polymers (both spin coated and dip coated) and on control 2D glass coverslips. After overnight attachment, the substrates were transferred to fresh six-well plates. As defined earlier, this time point was designated as day zero for quantitation. In order to determine the number of cells attached to the film after transfer to new six well plates, the wells of the old plates were subjected to trypsinization and detached cells were collected in MEM medium. The cell count of this fraction was deducted from the total number of cells seeded at the beginning of the experiment to obtain the number of cells on the polymer samples transferred to new wells on day zero. Cell counting was carried out with trypan blue on hemocytometer. For each time interval (day 0, 1, 3, 4, 5, 6 and 8), triplicate samples (separate well plates for each time interval) were examined to obtain at least six measurements.
5.3.8 RNA isolation and PCR Analysis

To study the gene expression profile of cells grown on 3D PLGA and 2D samples, cells from the various samples were harvested using 0.25% trypsin-EDTA solution. Total RNA from these cells was then extracted using TRIzol reagent (1 ml reagent per 1 million cells approximately) (Invitrogen). Purity and quantity of RNA was measured with Nanodrop spectrophotometer (Thermo scientific Inc). Using 1 µg RNA and random primers, c-DNA was synthesized after incubating the RNA at 68°C for 10 mins with superscript RT (AB applied biosystem) at 42°C for 60 min. PCR amplification of targets of interest was followed with hot start green Taq DNA polymerase (Promega, Madison, WI) for each set of selected primers. Amplification conditions and expected amplicon sizes of each of the primer set used are described in Table 2. The amplified PCR products were separated on 2% agarose gels (Agarose I biotechnology grade, Amresco, OH) and stained with ethidium bromide (Sigma Aldrich). Resolved gels were visualized and captured with BIORAD UV imaging system.
Table 2. Primer sequences, Melting temperature (Tm), and expected amplicon sizes in bp

<table>
<thead>
<tr>
<th>Primer/Marker Names</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
<th>Tm (°C)</th>
<th>Amplicon size (base pair)</th>
<th>Reference</th>
</tr>
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<tr>
<td>GATA3</td>
<td>5'-GTGAACTGTGGGCACACCTC-3'</td>
<td>5'-TTTCGTTTCTGGTCTGAGATG-3'</td>
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<td>311</td>
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<td>5'-CTGAACCTCCTGGTGACCG-3'</td>
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<td>202</td>
<td>NM_00102420</td>
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<tr>
<td>INTEGR4</td>
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<td>5'-TCAAATGCGGCTCCTCA-3'</td>
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<td>371</td>
<td>NM_001005731</td>
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<tr>
<td>B2MG</td>
<td>5'-GGTTTCATCCATCGACATG-3'</td>
<td>5'-AAGCGAGCCAGCAGAATTGG-3'</td>
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<td>CALLA</td>
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<td>52</td>
<td>324</td>
<td>NM_007289</td>
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5.3.9 Doxorubicin treatment on MCF-7 cells

The effect of doxorubicin, an anti-cancer chemotherapeutic agent, on the proliferation of cells cultured on 3D PLGA and 2D cover glass environment was examined using the Alamar blue cell viability assay. Unlike the other assays, a total of 5x10³ MCF-7 cells were plated per well of a six-well plate for this assay. The reason to seed with a smaller number of cells was to allow them to stay in log phase for a longer period of time without reaching confluence. The desired drug concentrations (0.5 µM and 5 µM of doxorubicin hydrochloride) were prepared by diluting the drug stock solution in MEM cell culture medium. The first doxorubicin treatment was given after five days of cell culture. For untreated control samples, dimethyl sulfoxide (DMSO – Sigma Aldrich, volume equivalent to 5 µM doxorubicin) was used. Fresh media containing the appropriate concentration of the drugs was replenished every 72 h. The Almar blue assay
was performed according to manufacturer protocols (Invitrogen, Carlsbad - CA) on days 5, 8 and 11 post plating the cells. Briefly, 100 µL of the Alamar blue reagent was added to cultures; following incubation for 4 h at 37 °C, treated samples were pipetted out in triplicates into a fresh 96-well black plate. The absorbance was recorded using a FLx800 Fluorescence microplate reader (Biotek Instruments, Inc). In relation to DMSO treated control samples, the percentage (normalized) of viable cells for the drug treated samples was calculated.

5.3.10 Statistical Analysis

All experiments were carried out in triplicate or as indicated otherwise. Data is expressed as mean ± standard deviation. Statistical significance was determined using Students two-tailed paired t test or Mann-Whitney Rank Sum Test. Differences were considered statistically significant for values of p ≤ 0.05.
5.4 Results and discussion

5.4.1 Engineering PLGA based synthetic scaffolds using breath figure process

Our current understanding of tissue form, function and their aberrant pathologies has evolved from 2D cell cultures which often behave very differently from the 3D microenvironments of cells in animal model studies. It is becoming more and more apparent that the former approach is limited by considerable differences in morphology and differentiation program when cells are cultured in 2D substrates. Contrarily, animal studies are not only extremely time consuming but very expensive as well. 3D culture techniques combine the flexibility and speed of tissue culture with tissue architectural cues afforded in vivo. It is no surprise therefore; that several studies have attempted to build 3D models for mammary morphogenesis using various types of extracellular matrix (ECM) components. However, to-date only a few studies have tried to use purely synthetic scaffolds for mammary morphogenesis. We describe here, our efforts to design and engineer synthetic 3D scaffolds that faithfully recapitulate the in vivo microenvironment of breast tissue. Our objective was to choose biopolymers that can influence better cell adhesion, polarity and induce cellular differentiation of mammary cells. Other considerations were biocompatibility and biodegradability, topography and mechanical properties. The porosity of the biomaterial was another major factor that was varied to assess how it influenced 3D growth. Mindful of these requirements, we describe here the engineering of PLGA breath figure based thin polymer scaffolds and evaluation of their potential to support 3D growth of the mammary epithelial cell line MCF-7. Besides the biodegradability of PLGA, the simplicity of forming pores in such materials
using the breath figure method make these structures viable candidates for scaffold construction.

SEM characterizations of the spin coated breath figure porous PLGA film revealed that the surface of the polymer (Figure 28A) was filled with the presence of self-assembled honeycomb-like pores throughout the large surface area of the sample. The average pore size measured was \(4.8 \pm 2.6 \mu m\). The formed micro-porous polymer matrix may be an ideal model system which closely resembles the stiffness of ductal architecture and the tumor microenvironment \textit{in vivo}. The pores on the surface provide large surface area for better cell adhesion whereas the presence of inter-connected pores allows infiltration of cells and diffusion of organic nutrients into the bulk polymer to induce cell-matrix interactions \(^{99}\). During the breath figure process, pore formation is not restricted to the surface alone. Instead it is also prevalent in the bulk phase as a multilayered porous network. A cross-sectional view in Figure 28B better illustrates the nature of the inter-connected porous structure. As stated by Bolognesi and coworkers\(^{44}\), formation of such multi-layered porous structure in the bulk phase is mainly due to the interfacial energy interaction which allows the water droplets to sink into the polymer film during the evaporative cooling of breath figure process. For the PLGA-dichloromethane system, we have reported earlier the formation of such porous structures\(^{44, 58}\) in the bulk polymer. Figure 28C and 28D show the SEM images of surface and side view of the spin coated non-porous PLGA film. As expected, the surface of the film was completely lacking porous structures. This may be due to the fact that the non-porous film was fabricated by purging nitrogen gas (< 10% humidity) into the spin coating chamber. Under the inert atmosphere, the solvent evaporates out quickly and creates a film with no condensation of
water droplets. When compared to the opaque natures of the 3D breath figure PLGA, the non-porous 2D films obtained were transparent with virtually no pores or ridges on the surface.

Figure 28. Scanning electron microscopy (SEM) images of spin coated porous and non-porous PLGA films (A) and (B) show the surface and cross-sectional view of spin coated porous PLGA. The breath figure PLGA coatings obtained were opaque and highly porous (average pore size 4.80 ± 2.6 µm) in nature and the side view showed the presence of inter-connected and multi-layered pores in the bulk polymer phase. (C) and (D) show the surface and cross-sectional morphology of spin coated non-porous PLGA film.
5.4.2 Porous architecture and roughness of the spin coated PLGA scaffolds allows stronger adhesion and enhanced morphogenesis of breast epithelial cells

To further assess if the porous architecture of the spin coated PLGA scaffolds is indeed a better substrate to support 3D growth, MCF-7 cells were cultured on spin coated PLGA, non-porous PLGA film, or glass cover slips for 7 days and stained with toluidine blue. Morphological assessment and acinar growth was scored to compare growth amongst the three surfaces (3D spin coated PLGA, 2D spin coated PLGA and 2D coverslips). Phase contrast imaging showed that cells adhered well and spread as a thin single layer of cells on the glass surface (Figure 29A) with little if any overlaying on top of each other or cell aggregation on the surface. The same was true for growth on the 2D non porous PLGA film (Figure 29B). On the other hand, very interestingly, cells grown on the spin coated PLGA scaffolds adhered well and demonstrated distinct three dimensional growth demonstrating round and well-developed cell aggregates with features of branching morphogenesis (shown with arrows) and lobulo alveolar growth (shown with asterisk), (Figure 29C and 29D). These morphological features are characteristic of typical human luminal epithelial cells in vivo. In contrast, cell shape in 2D systems was observed to be stretched and spindly and the lobular units in 3D PLGA varied in size with a range of 100–300 µm. Notably, even though the volume of toluidine blue solution used for staining of 2D and 3D samples was the same, the images of Figure 29 suggest that the uptake of dye solution is much higher in the porous spin coated samples as compared to 2D flat samples (cover glass and non-porous PLGA). Perhaps, the large surface area of the porous PLGA allows accommodation of larger cell
aggregates in a confined space which results in increased uptake of staining solution. In the case of flat substrates, cells

Figure 29. Morphological analysis of MCF-7 cells cultured for 7 days on 2D controls and 3D spin coated PLGA. Staining procedure: Toluidine blue (Scale bar indicates 100 µm).

(A) Toluidine blue staining shows monolayer growth of cells in 2D cover glass.

(B) represents the staining on 2D non-porous PLGA film. The cell growth pattern was more or less similar to 2D glass with little aggregation of cells on the surface. The flat film does not render micro-environmental roughness characteristics to support cell proliferation and differentiation. Instead, the cells adhered and proliferated as a monolayer structure.

(C) and (D) shows cell growth on porous spin coated PLGA. The growth was clearly three dimensional with large number of cell aggregation and clusters. Formation of elongated branched ductal and alveolar structure (Arrowhead indicates duct-like structure, asterisk indicates lobular-alveolar structure) are typical characteristics of mammary gland.
attach and proliferate as a single layer of sheet along the surface and thus do not accumulate as much dye. SEM analysis further substantiates our observations from phase contrast microscopy. The SEM images of Figure 30 show the monolayer distribution and elongated shape of MCF-7 cells growing on glass cover slips and on non-porous PLGA film surfaces (Figure 30A and 30B). However, in the case of 3D spin coated PLGA scaffolds, cells were arranged as clusters and grew on top of each other as seen in Figure 30C (low magnification) and Figure 30D (high magnification). Overall, the comparative analysis of growth in 2D and 3D spin coated PLGA suggests that the presence of porosity in the spin coated PLGA greatly influences the cell behavior and directs the cell to sense the surrounding micro-environment. These increased cell-cell and cell-polymer matrix interactions are able to promote proliferation and differentiation of MCF-7 cells into defined three dimensional structures. However, when cultured for longer durations, MCF-7 cells were attached mainly on the surface of the 3D spin coated PLGA utilizing the roughness on the topography and large surface area of micro-cavities, and no significant growth of cells was observed in the bulk PLGA phase. This may be due to the fact that the average pore size of the spin coated PLGA (4.8 µm) was less than the size of MCF-7 cell (6-8 µm) and hence allowed only nominal growth inside the scaffolds.
Figure 30. SEM of MCF-7 cells cultured on 2D systems and 3D spin coated PLGA. (A) and (B) shows the surface topography of fixed MCF-7 cells cultured on 2D cover glass and 2D flat PLGA film, respectively. (C) and (D) show low and high magnification of cells grown on 3D spin coated PLGA.
Figures 31 (A) and (B) show low and high magnification SEM images of dip coated porous PLGA. Unlike spin coated porous PLGA, the dip coating produced larger pores and the pore size was in the range of 5-30 microns. The average pore size measured was 15.28 ± 5.2 µm.

Figure 31 C shows the comparison of percentage population of pores measured for both spin coated and dip coated samples. Using Image J software, we recorded 300 pores from at least 5 SEM images. Each pore was measured for pore size. Compiled average pore size and distribution in over the range 0 – 30 µm are shown in the plot. More than 65% of pores were in the range of 12-30 microns for dip coated 3D samples. In contrast, 50% of pores were in the range of 4-12 microns for the spin coated 3D samples.
5.4.3 Endogenous honeycomb pattern of the dip coated PLGA facilitates branching morphogenesis

To assess if increasing the pore size would allow more robust growth in the bulk phase, we modified the coating procedure in the breath figure process. Instead of spin coating where the solvent is quickly evaporated off, we sought to dip coat the substrate so that the solvent is vaporized slowly in the presence of humid air. After dipping the glass substrate into the polymer solution, the substrate was kept in a slanted position under high humidity. By passing the humid air for longer period of time (~ 0.5-1 h), the evaporation of solvent from the polymer surface was slow and induced more condensation of water droplets and thereby, increased the pore size. Figure 31A and 31B show low and high magnification SEM images of dip coated porous PLGA scaffolds. Compared to spin coating, the average pore dimension measured was 15.28 ± 5.2 µm. Figure 31C shows the pore size distribution plot for both 3D spin and dip coated PLGA samples. For dip coated porous PLGA, more than 65% of pores were formed in the size range of 12-30 µm. Contrarily, the spin coated porous PLGA exhibited 50% of pores in the range of 4-12 µm. Similar to the spin coated porous PLGA, cells seeded on the dip coated porous PLGA grew into well-defined 3D structures and showed clear mammary epithelial cell morphology (Figures 32A and 32B). Notably, the dip coated scaffolds supported more robust growth with proportionally more acinar-like structures with the morphology of differentiated structures (lobular-alveolar units) (shown with asterisk) which were greater in 3D dip coated PLGA than in the 3D spin coated PLGA. We also confirmed through SEM (Figure 32C) that the cells not only form aggregates but also invade and infiltrate the bulk PLGA. To assess whether the synthetic PLGA scaffolds
steer the growth of MCF-7 cells towards acinar morphology and induced branching morphogenesis in the dip coated 3D culture systems, cells plated in 2D cover glass and non-porous PLGA film were compared with cells grown on dip coated PLGA scaffolds. After 8 days in culture, cells grown on dip coated PLGA scaffolds formed cysts like structures, which were spherical and glandular in appearance and contained a hollow lumen with many cellular processes arising from the basal surface of the cystic growth. The term “branching,” is being used to describe the tubular processes as they resemble the structures that form during mammary morphogenesis in vivo. These cultures also showed lumen-containing lobular structure. 2D cultures on the other hand showed only monolayer growth except at the edges, where occasionally some overlaying of cells was observed. Overall, our data shows that dip coated PLGA scaffolds with larger pore sizes support more robust 3D growth of the mammary cells (1.88 times larger lobular-alveolar size) in comparison to spin coated scaffolds. Notably, the branching phenotype of the cyst-like structure was observed as early as day 5 of culture but was strongest around day 8 - 10. However, due to substantial increase in cell death by day 10, culture timings were limited to 8 days for most studies where quantitative studies were undertaken.

Quantitation of duct-like and lobular-alveolar structures obtained from different batches of experiments (from 3 experiments) and measured from 15 phase contrast images showed that the average percentile of duct-like structure (Figure 32D) obtained for both 3D spin coated and 3D dip coated PLGA was 36.8% and 33.9%, respectively. Although, the difference in percentage of ductal outgrowth between spin and dip coated 3D PLGA was not statistically significant (p >0.05), this difference was highly
significant when 3D out-growths were compared to percent ductal growth in 2D cover glass (p < 0.01). We next asked could this significantly different response stem from differences in ECM crosslinking and stiffening within the two environments.

**Figure 32** (A) and (B) show low and high magnification of morphological development of MCF-7 cells cultured on dip coated porous PLGA. Toluidine blue staining shows the robust growth of cells differentiated into duct-like (shown in arrows) and lobular-alveolar structures (shown in asterisk). Scale bar denotes 100 µm. (C) shows the SEM image of corresponding 3D morphology of MCF-7 cells fixed and gold coated after 7 days of cell culture. (D) Quantitation of percent duct like structures in 2D cover glass, spin and dip coated cultures. Compared to 2D cover glass, ductal growth is highly significant on 3D spin and dip coated PLGA scaffolds (p<0.0001, Dunnett's Multiple Comparison Test). Experiments were conducted three times and for each experimental condition, five random fields were imaged and analyzed for % ductal growth (% ductal growth = [(No. of ducts)/(No. of ducts + No. of lobular-alveolar structures)]*100).
5.4.4 Pattern of K18 and E-cadherin expression confirm the polarized ductal/acinar structure of the 3D outgrowths in dip coated PLGA scaffolds

ECM signaling has been shown to drive the cellular changes which sculpt tissues and organs during embryogenesis\textsuperscript{116, 117}. For the same reasons, abnormalities in the mechanical environment of epithelial tissues can contribute to their malignant transformation and progression\textsuperscript{118}. Conversely, exposure of cancer cells to micro-environments of a healthy tissue should help their transition to more normal differentiated phenotype\textsuperscript{119}. To investigate this issue, we asked whether microfabricated PLGA scaffolds with relatively large porous matrices would provide an environment where mammary cells could lay down their own matrix, make their own autocrine and paracrine regulatory factors and steer the growth of epithelia in a stereotyped pattern to form ducts and luminal structures even in the absence of differentiating agents.

In order to test whether the porous matrix of the dip coated PLGA scaffolds supported branching morphogenesis and luminal growth, confocal analysis of 2D and dip coated 3D scaffolds was performed (Figure 33A). Ductal/acinar attributes of the outgrowths were ascertained with expression of lineage specific ductal and luminal markers K18 and distribution of epithelial-Cadherin (E-cadherin). These proteins were revealed by green fluorescence. The relatively more porous dip coated matrix showed intense K18 positive ductal branching outgrowths with lumens (Figure 33A Panel b and d) as compared to the pattern of growth in the 2D control cover glass systems (Figure 33A Panel a and c). Furthermore, consistent with our predictions, significant deeper ingrowth of the branched cystic structures was recorded in the dip coated scaffolds (p=0.02, Mann Whitney test, Figure 33B).
Figure 33. 3D scaffolds support more robust lobulo-alveolar growth with or without mammary differentiating agents. Scale bar indicates 100 µm.

Figure 33A. Confocal imaging of K-18 stained MCF-7 outgrowths on 2D glass coverslips (panels a1 to a3 and c1 to c3) and dip coated porous PLGA 3D scaffolds (panels b1 to b3 and d1 to d3) after a week of culture in the presence (rows a and b) or absence (rows c and d) of differentiating agents as described in the methods sections. Lobulo-alveolar acini and ductal outgrowths occupied almost whole of the 3D PLGA scaffolds but were not observed in 2D glass coverslip cultures. The structures encircled with red broken dashes identify lobulo-alveolar acini. The stained specimens were imaged simultaneously at two different excitation wavelengths (405 and 488 nm) using a single krypton/argon laser to obtain individual optical sections at high resolution in sequence through the thickness of the specimen. Column 1 represents a z-series projection of nuclei stained with DAPI (blue). Column 2 represents the corresponding z-series projection of lobulo-alveolar acini stained with K-18 and visualized using Alexa 488 secondary antibody (green). Column 3 represents a z-series projection overlay of DAPI and Alexa 488 staining. The no antibody controls are shown as insets in the projection overlay panel. Photomicrographs are representative of at least three different experiments.
Figure 33
Figure 33 B. Histomorphometric quantitation of optical thickness of the outgrowths on 2D and 3D dip coated PLGA scaffolds stained with DAPI and K-18. $n = 4$ fields of view from independent experiments. The average thickness of 3D outgrowths was found to be 1.5 times that of cells cultured in 2D systems. This was validated by the significantly different medians ($p=0.0286$, two tailed Mann Whitney test) of the two groups of outgrowths.

Figure 33C and 33D. Cells grown on 3D PLGA scaffolds show strong cell adhesion and maintain proper polarity compared to the control cells grown on glass coverslips. Figure 33C exhibits confocal laser scanning microscope images of E-cadherin/DAPI double immunofluorescence-stained mammary outgrowths from 3D PLGA scaffolds, showing lumen containing acini with distinct apico-basal expression of E-cadherin. Figure 33D shows E-cadherin/DAPI double immunofluorescence-stained mammary outgrowths from 2D glasscover slips. Photomicrographs are representative of two independent experiments.
As detailed in the methods section, the average pattern thickness of the branching structures was generated by stacking and color-coding multiple (~15 – 50 depending on the thickness of the growth) images of a lobular unit stained with K18 and DAPI. Based on these results, we also examined how larger pore size of the matrix may affect ductal polarity of MCF-7 outgrowths in the engineered scaffolds. Our rational to particularly investigate changes in polarity was based on earlier reports that have shown that in the right context, either due to an injurious insult or environmental perturbations, the breast cancer epithelium may become activated and primed for induction of epithelial plasticity and undergoes Epithelial-Mesenchymal Transition (EMT). This transition is usually accompanied by loss of E-cadherin expression and apico-basal polarity. However, if, our hypothesis that cystic growth in the porous environment of the dip coated PLGA may allow for deposition of a cell’s own ECM and growth factors, then one would expect to see no loss of E-Cadherin or apico-basal polarity. Indeed our data on the expression of E-cadherin revealed that ductal/acinar outgrowths maintained an epithelial phenotype including correct apico-basal polarity and cell-cell adhesions (Figure 33C and 33D). Quantification of the dip coated and spin coated cultures showed that more than 80% of the area of the outgrowths had normal, ductal morphology, as opposed to less than 5 % in 2D non porous films that too mostly at the edges of the 2D films. Notably, contrary to our expectations, addition of differentiating agents to cultures growing under 3D conditions did not show significant differences in acinar growth with or without the differentiating agents (Figure 33A compare panel b and d). However, to gain a better understanding of the subtle differences in bio-molecular nature of the differentiated phenotype of the 3D cystic acinar outgrowths that may have been missed by our gross morphogenetic analysis,
RT-PCR analysis of some known lineage specific mammary differentiation genes was undertaken.

5.4.5 Distinct phenotype of 2D and 3D outgrowths at the molecular level

As shown in Figure 34, RT-PCR analysis identified a significant upregulation of GATA3, EMA and Integrin β4 transcripts mainly in the 3D outgrowths. Expression of all three of these genes has been associated with luminal differentiation, further confirming that outgrowths of breast cancer cells in a 3D environment acquire a more differentiated phenotype. However, contrary to published reports that suggest EMA expression is high in ductal/acinar outgrowths, we observed its expression to be high under both 2D and 3D culture conditions. An explanation for its expression in both types of culture conditions is that EMA is also known to be expressed in bipotent progenitor stem cells suggesting that both luminal and more spindly mesenchymal cells may express it. We therefore considered the possibility that in addition to exhibiting differences in inducing luminal lineage genes, the 3D PLGA matrices may also differ in their ability to support progenitor cell growth. To address this issue we looked for expression of stem cell specific markers. As no mammary stem cell specific markers are known, we sought to study the expression of NOTCH-1 based on their role in hematopoietic stem cell renewal and differentiation. Consistent with our previous observations, the expression of NOTCH-1 was seen only under 3D growth conditions, further suggesting that outgrowths from 2D and 3D culture were indeed distinct from one another at the molecular level and that they differed in their ability to support progenitor cell differentiation. Further evidence of molecular differences in the outgrowths came from the study of the expression of myoepithelial marker. As shown in Figure 34 expression of myoepithelial
marker CALLA decreased in response to the addition of differentiating agents; although, contrary to our expectations, this decrease was noted in both 2D and 3D outgrowths.

**Figure 34.** Expression of genes involved in mammary cell differentiation and some stem cell markers is enhanced in cells grown on 3D PLGA scaffolds in the presence or absence of differentiating agents. Expression of mesenchymal genes is reduced in response to differentiation. Representative photomicrographs of semi-quantitative RT-PCR amplification gels of mammary differentiation, stem cell markers and mesenchymal genes from total RNA isolated from MCF-7 cells cultured on 2D (lanes C₁ C₂), Spin coated (lanes S₁ S₂) and dip coated (lanes D₁ D₂) PLGA scaffolds. Lanes C₁, S₁ and D₁ represent samples grown without any differentiating agents and lanes C₂, S₂ and D₂ represent samples grown with differentiating agents.
5.4.6 Growth proliferation of MCF-7 cells on 3D PLGA and 2D substrate

The morphological changes triggered by the interaction between the microenvironment of a porous 3D matrices and MCF-7 cells led us to ask whether the spin and dip coated PLGA scaffolds can support the recovery and proliferation of cells seeded after trypsinization just as cells in 2D do. To address this question, we seeded $2.5 \times 10^4$ MCF-7 cells/well (Figure 35) on preconditioned porous PLGA samples (both spin coated and dip coated) and on the control glass coverslips. The plates were observed daily under a standard light microscope. It is an empirical task to look for cell proliferation on the 3D scaffolds since the cells not only adhere tightly onto the topography of these slightly opaque surfaces but also migrate into the porous architecture of these scaffolds, necessitating repeated trypsin treatment to release all the cells before counting. It may be noted that detaching cells from the polymers required twice the amount of trypsin than what was required to remove cells from the 2D cover glass. Quantitation of number of cells following 8 days of culture demonstrated that 3D dip coated and spin coated PLGA had a 56-fold and 64-fold increase in cell density at the end of the 8 day culture period, respectively. As expected, growth was even higher in the case of 2D cover glass, where a 74-fold increase in cell density was observed.
Figure 35. Growth proliferation kinetics of MCF-7 cells on 3D dip coated, 3D spin coated and 2D glass cover slips. The overall cell growth was higher in 2D system in comparison to 3D polymers after 8 days of cell culture. Significant statistical difference of cell proliferation between 2D and 3D spin coated samples (student t-test $^*p<0.05$). For day 4, no statistical difference observed between 2D and 3D dip coated samples ($^#p>0.05$). However, for day 8 proliferation, there was a significant difference in the growth kinetics between 2D and 3D systems with high statistical difference observed between 2D and 3D dip coated samples ($^{+}p\leq0.01$). Initial cell seeding density = 25000 cells/substrate. Experiment was conducted in triplicate and data plotted as mean ± SD.
Although, we seeded a very small number of cells on day zero, clusters of proliferating MCF-7 cells were formed routinely on these scaffolds. We speculate that both the interaction of cells with the microenvironment of the porous matrix and the cell density provide a conducive environment for cells to proliferate initially. This is clearly illustrated in the graph in Figure 35. However, even though the graph reveals a progressive increase in the number of cells as a function of time supporting the notion that trypsinized cells seeded onto the 3D scaffolds are able to recover and grow, the growth at later time points starts to plateau and eventually starts to slow down (growth on day 5 and day 6). This may be because of the honeycomb nature of the scaffold and the cell density being in the low range at earlier time points. These attributes allow MCF-7 cells to possess morphological features of regularly dividing spindly cells instead of their expected growth as clusters in 3D. However, once they started to grow as clusters at later time points and cell matrix communications are established, cell proliferation slows down to allow differentiation to commence (as evidenced from our other experiments). In contrast, cells on 2D continued to proliferate all through the duration of the experiment. Our data suggests, trypsinized MCF-7 cells are able to recover in the 3D matrix of the spin and dip coated PLGA and that cell density and cell matrix interactions together influence proliferation and differentiation of cells in 3D PLGA scaffolds.

5.4.7 Doxorubicin treatment for cells grown in 2-D monolayer and 3D samples

We observed that culturing MCF-7 cells in 3D scaffolds produces distinctly different proliferative and differentiation responses than when cultured on standard 2D glass coverslips. Additionally, the move to a 3D culture platform yields very different transcription levels of some of the known differentiation genes. In order to assess, how
this difference in bio-molecular behavior in a 3D environment affects the response of these cells to pharmacological agents, we treated MCF-7 cells growing on 2D cover glass, spin and dip coated scaffolds with doxorubicin (0.5 and 5.0 µM). Doxorubicin is an anthracycline antibiotic that is currently considered to be one of the most effective agents in the treatment of breast cancer\textsuperscript{120}. The response of cell proliferation to the drug treatment was evaluated using the alamar blue cell viability method\textsuperscript{121}. From the absorbance values measured, the percentile cell viability was determined by normalizing the data with respect to the controls (DMSO treated, but without exposure to doxorubicin). As can be seen from Figure 36A, the response of MCF-7 cells growing as a 2D monolayer and exposed to 0.5 µM doxorubicin is such that by day 3 of treatment there are only 20% of viable cells present as in the control 2D system. For the 3D culture, however the effect of doxorubicin is less drastic and there is \textasciitilde 55% survival in comparison to the 2D controls. When the growth response to doxorubicin in 2D is compared to growth response in 3D, the difference is statistically significant (p < 0.05) and is to be anticipated from the reduced proliferation and enhanced differentiation activity in a 3D environment. Moreover, cells in 2D grow homogeneously with an adequate supply of nutrients and no barrier for diffusion of drug components. Additionally, the reduced sensitivity to doxorubicin in the 3D PLGA environment is influenced by the porous microenvironment which replicates the \textit{in vivo} physiological milieu of heterogeneity ranging from the reduced diffusion of nutrients, hypoxia and increased cell-cell and cell-polymer matrix interactions\textsuperscript{106}. Overall, our results correlate well with previous reports that have shown that tumor cells grown in 3-D are more resistant to chemotherapy agents as compared to 2D cultures with PLGA\textsuperscript{97,103}. However,
treatment with higher concentrations of doxorubicin (≥ 5 µM) (Figure 36B) showed little difference in behavior because at these high concentrations the drug was immediately toxic to cells.

**Figure 36.** Doxorubicin treatment for MCF-7 cells grown on 2D control and 3D PLGA scaffolds

Percent cell viability plotted over time after exposing the MCF-7 cells cultured on 2D controls or 3D scaffolds every 72 h with 0.5 µM (A) or 5 µM (B) doxorubicin concentration. Viable cells were measured using the standard alamar blue staining procedure. In comparison to 2D controls, both spin coated and dip coated PLGA scaffold systems exhibit resistance to the doxorubicin. At the end of day 3 treatment, 55% of plated cells were viable in 3D group with respect to only 20% viable cells in 2D controls. Statistically, the obtained mean values were significantly different between 2D and 3D samples (students t-test *p < 0.05). Increasing the drug concentration level to 5 µM shows minimal toxicity effect between 2D and 3D samples. However, cells are more viable in 3D dip coated PLGA than 3D spin coated PLGA. This data suggests monolayer growth pattern in 2D cell culture facilitates increased uptake of drug in comparison to 3D cell culture, where multi-layered formation of cells probably resists drug diffusion.
5.5 Conclusions

In summary, this study provides quantitative understanding of the role of synthetic microenvironments in altering cellular behavior, in particular changes in proliferation, differentiation and response to pharmacological intervention in 2D and 3D environments. The data indicates that synthetic porous PLGA scaffolds provide a three-dimensional milieu that resembles the *in vivo* mammary environment. Our studies have shown that incorporation of breath figure PLGA scaffolds for culturing mammary cells would be of considerable benefit for a comprehensive understanding of their cellular behavior, differentiation and response to drug treatment. Use of PLGA polymer in the biomedical field is well known for drug delivery and tissue engineering applications because of its convenient to modify properties including degradation characteristics. Moreover, as PLGA degrades hydrolytically and releases monomers that can be absorbed into the body metabolism with no toxic remnants, our 3D scaffolds can be safely used in regenerative medicine field.
Chapter 6 - A Novel Micro-patterning Of Hydrogels Using Breath Figures As Template

6.1 Summary

This study describes a novel and cost-effective approach of micro-patterning hydrogels using a breath figure polymer film as a master template. The breath figure technique was employed to produce a mono-dispersed porous polymer film through the evaporation of a polymer solution (9:1 weight ratio of Poly (lactic-co-glycolic acid) and Poly(ethylene glycol)) under constant humid conditions. Using hydroxyethyl methacrylate (HEMA) as a monomer, the precursor solution of the hydrogel was prepared and cast on top of the breath figure template and was allowed to polymerize overnight. Subsequent dissolution of the template in an organic solvent lead to the formation of the reverse template of breath figures on the poly (HEMA) hydrogel surface. The resultant micro-patterned bead-like formation on the hydrogel surface is of the order of the original breath figure pore diameter (~1.5 µm) and the pore depth, as assessed by scanning electron microscopy and confocal microscopy. Furthermore, the simplicity and versatility of the breath figure template was further explored with the incorporation of iron nanoparticles to make magnetically responsive polymer films. Through the casting of the hydrogel precursors onto the iron-filled breath figure template, the iron-particles were selectively embedded on the patterned PHEMA surface.
In addition, similar patterning was successfully obtained for various biopolymers such as chitosan, carboxymethylcellulose (CMC), gelatin and poly (vinyl alcohol) (PVA) through this simple casting procedure. This newly developed breath figure based templating approach is simple to perform with a high fidelity over a large area of the substrate. Our findings indicate that the breath figures based micro-patterning of hydrogels and biopolymers may be an effective and attractive biomaterial to develop cell and protein micro-patterning substrates, tissue engineering, cell-surface interaction studies and biosensors.

6.2 Introduction

Biological materials with topologically patterned micro or nanostructures have received an immense interest in the field of micro-fabrication technology\textsuperscript{122-124}. For example, a selective incorporation of the protein molecules on spatially oriented substrates has been reported as an important tool in the applications of cell biology, biosensors, and various biomedical diagnostics\textsuperscript{123, 125-127}. Mostly, polydimethyl siloxane (PDMS) based soft lithography or colloidal crystals templating are used to either create or transfer patterns to the biological materials such as Hydrogels, Matrigel, Agarose gel and so on\textsuperscript{105, 126, 128-130}. The major advantages of using soft lithography via a PDMS mould are the ease of processing and non-requirement of a complex equipment setup to produce patterns on the materials\textsuperscript{131}. However, a master templating tool is required to use soft lithography and may involve multiple steps to achieve a desired pattern. The template is normally generated from the photolithography masks which require an expensive setup and sophisticated clean room environments\textsuperscript{122}. Therefore, the micro-
patterning of the biomimetic substrates through a simple and a cost-effective approach is highly desirable.

Among several polymers investigated for biomedical applications, synthetically derived polymeric hydrogel, poly(hydroxyethyl methacrylate) (PHEMA), is being extensively studied as a biomaterial particularly in the field of contact lenses, tissue engineering, medical implants, drug release systems, biosensors and microfluidic devices. PHEMA is a chemically cross-linked, hydrophilic, water insoluble polymer which swells significantly in water (depending on its cross-linker concentration). These network polymers have gained a wide acceptance as biocompatible materials as its biomimetic properties can be easily altered by applying external stimuli such as temperature, pH and ionic strength. This enables the PHEMA hydrogel to be used in various biological systems to restore and regenerate functional tissues.

Creation of surface-patterned PHEMA hydrogel is of the interest in this paper. In the literature, Guvendiren and coworkers have reported the significance of surface patterned PHEMA hydrogels in controlling stem cells morphology and differentiation spatially in comparison to flat hydrogels with no patterns on the surface. They used gradient PHEMA masters to replicate patterns with lamellar and hexagonal shape on PHEMA gels through the PDMS replica molding process. In another study, by combining photolithography and soft-lithography, Yu and coworkers have prepared the topographically patterned PHEMA hydrogels and functionalized the surface pattern features with binding of proteins selectively. Most of these studies mentioned in the literature have used either photolithography masks or soft-lithography templates to pattern micro-structures on the hydrogel surface.
In this study, we have utilized breath figures as an initial templating tool, which is based on a simple bottoms-up approach in which water droplets self-assemble and produce a porous structure in the polymer. The creation of the porous template using the breath figure process has been explored for a variety of polymers and it is a well-known process due to its simplicity and convenience of use. Recently, Galeotti and coworkers have explored the breath figure patterned polystyrene films as a template to patterning silk fibroin films and have shown spherical-shaped bumps on the silk film. However, the work described in the current study is the first attempt of micro-patterning the surface of a PHEMA hydrogel and other soft materials like Chitosan, Cellulose, Gelatin, Poly (vinylalcohol) using breath figures as templates prepared from biodegradable polymers (PLGA and PEG).

This study describes a single step process for micro-patterning a PHEMA hydrogel surface with a monolayer arrangement of bead-like structures using the breath figure PEG/PLGA film as a template. The breath figure PEG/PLGA film was obtained by spin coating a PEG/PLGA solution (1:9 weight ratio) under high humidity conditions. Using the breath figure template, we were able to embed nanoparticles on the tip of the protrusions of the hydrogel’s patterned surface using magnetic iron nanoparticles (Reactive Nanoscale Iron Particles –RNIP) as a model nanomaterial. Moreover, similar patterning was successfully achieved in various biopolymers such as chitosan, hydrophobically modified chitosan, gelatin, carboxymethyl cellulose and polyvinyl alcohol. The topographically micro-patterned soft materials have potential applications in the field of protein micro-patterned substrates, drug delivery, tissue engineering, cell-surface interaction studies and biosensors.
6.3 Materials and Methods

6.3.1 Materials

Poly (D,L-lactide-co-glycolide) (PLGA 50:50) polymer (Resomer RG 504 Mₕ=56000 & Inherent viscosity = 0.56 dl/g) was purchased from Boehringer Ingelheim Chemicals Inc, USA. Methylene chloride (organic solvent - ACS grade) was obtained from Fisher Scientific, USA. Reagents for hydrogel synthesis [2-hydroxyethyl methacrylate (99% solution); N,N’-methylene-bisacrylamide (MBA); N,N,N’,N’-tetramethylethylenediamine (TEMED); and ammonium persulfate (APS)] were purchased from Sigma/Aldrich (St Louis, MO). Reactive Nanoscale Iron particles (RNIP) were obtained from Toda Kogyo Corporation, Japan. All chemicals were used as received, without further purification.

6.3.2 Fabrication of breath figure patterned polymer film

Breath figure thin films were prepared using a spin coating procedure (spin coater model - WS-400-6NPP-LITE, Laurell Technologies Corporation, North Wales, PA). At a weight ratio of 1:9, polyethylene glycol (PEG) and poly (lactic-co-glycolic acid) (PLGA) polymers were dissolved in dichloromethane to obtain a total polymer concentration of 15% (w/v). A 24 mm glass coverslip, used as the substrate, was placed on the fragment adapter inside the spin coater. The fragment adapter was kept under vacuum to hold the substrate while spinning. Through an orifice on top of the spin coater, a constant flow of humid air was introduced, which was created by bubbling atmospheric air through distilled water. The relative humidity was maintained at 70% throughout the experiment and monitored using a hygrometer (Fisher Scientific). Approximately, 1 ml of the
polymer solution was dropped onto the substrate and spun to 2500 rpm for a 30-45 s duration. During the spin coating process, the solvent was allowed to evaporate in the presence of the introduced humid air in order to obtain a porous and opaque film. The films were dried for a day at room temperature prior to further analysis and use.

6.3.3 Preparation of PHEMA hydrogel precursors and patterning hydrogel materials

The PHEMA hydrogel precursor solution was synthesized as described by Diane and co-workers\textsuperscript{55}. Briefly, 0.0127 g of the crosslinker, MBA was dissolved in 1 mL of distilled water containing 100 µL of TEMED (accelerator of the free radical formation). This was followed by the addition of 1 mL of the monomer, 2-hydroxyethyl methacrylate (HEMA) and vortexed. Subsequently, a 0.25 mL aliquot of ammonium persulfate (APS-the free radical initiator) in distilled water (0.5 mol% with respect to the HEMA monomer) was added to the mixture and vortexed again for at least 30 s to obtain a homogeneous precursor solution. The precursor solution was prepared fresh for each batch of the experiments.

A stepwise fabrication procedure for patterning the poly (HEMA) hydrogel is illustrated in Figure 37. Hydrogel precursor solutions were cast directly onto the breath figure PEG/PLGA film and left to cure for at least 12 h at the room temperature. To obtain a thin film of a flat and uniform hydrogel, we placed a glass coverslip on top of the cast solution. This allows the precursor solution to be spread uniformly across the porous PEG/PLGA film surface. The reaction between the HEMA monomers and the crosslinker (MBA) typically follows redox pathways through a free radical polymerization mechanism\textsuperscript{55}. For all the experiments, the crosslinker MBA was used at a 1:100 molar
ratio of crosslinker to the monomer. The sample was then left overnight to allow the precursors to polymerize. The resultant hydrogel appeared transparent to the naked eye. To obtain the patterned hydrogel, the entire materials (hydrogel cast on top of the PEG/PLGA film) were immersed into dichloromethane for 30-60 s. This induces the dissolution of the breath figure PEG/PLGA template and leaves the opaque pattern on the hydrogel surface. In order to micro-pattern both sides of the thin hydrogel film, we placed the precursor solution between two breath figure PEG/PLGA films (as shown in Figure 41).

We further extended the breath figure based microfabrication approach to incorporate nanoparticles onto the patterned-hydrogels surface. First, a uniformly dispersed 0.1% RNIP solution (RNIP consists of an elemental iron core (Fe) (50%) and a magnetite shell (Fe₃O₄) (50%)) was poured in the beaker containing the PEG/PLGA film affixed at the bottom. This led to the immersion of the PLGA-PEG breath figure film in the RNIP solution. The beaker was then placed into a bath sonicator (8890 Cole Parmer Ultrasonic Cleaner) for 5-10 minutes to allow the iron nanoparticles to fill selectively into the pores. After which, the surface of the film was extensively washed with 95% ethanol and distilled water to remove the excess nanoparticles bound to the surface. The nanoparticles filled PEG/PLGA film was dried for a day prior to further use. Similar fabrication procedures were followed to prepare patterned-hydrogel surface decorated with nanoparticles.
6.3.4 Characterization methods

The surface morphologies of the breath figure PEG/PLGA film and the micropatterned PHEMA film were characterized with a field emission scanning electron microscope (SEM) (Hitachi S-4800). Prior to the SEM imaging, all the samples were coated with a thin gold layer through sputtering (Polaron SEM coating system) set at 20 mA for the duration of 90 s. To image the cross-section of the pattern, the samples were frozen in liquid nitrogen for 3-5 s and broken, after which they were gold coated as well. To confirm the infiltration of the hydrogel precursor solution into the breath figure pores, we dissolved fluorescein salt (20 µg/mL) (Sigma Aldrich) into the hydrogel precursor solution prior to casting. The entire hydrogel and PEG/PLGA composite film was then imaged using a confocal microscope to see the pattern. The image was acquired using the 63x objective lens of a Zeiss LSM confocal microscope system (Carl Zeiss International, Germany) with green (488 nm) excitation filter sets.

6.4 Results and Discussion

The fabrication of micro-patterning on the biocompatible PHEMA hydrogel material is presented here using breath figure pores as a template. As depicted in Figure 37, the micro-fabrication of hydrogels is simple and consists of two steps. First, a thin porous polymer film is prepared (using spin coating) and subsequently, cast the hydrogel precursors to produce direct replication of the porous template into the hydrogels.
Figure 37. Schematic illustration of patterning hydrogels using breath figure template
We have previously reported that the breath figure method could be utilized to produce honeycomb-like porous structures, when biodegradable polymers such as poly(lactic-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG) are used at a weight ratio of 9:1. The mechanism of the breath figure formation and the experimental assembly described herein are cost-effective and highly reproducible. Briefly, the process involves the rapid evaporation of a polymer solution (dissolved in a high vapor pressure organic solvent such as dichloromethane) in a humid atmosphere. The evaporation of the organic solvent immediately cools the polymer solution surface which then induces the condensation of water droplets from the humid air stream. The temperature gradient arises from the condensed water droplets (due to the evaporation of solvent) induces convective motion of water droplets on the surface. The presence of convection current along with the laterally acting capillary forces further influences the water droplets to grow, stabilize and arrange into “islands” (hexagonal formation of water droplets). In the end, upon the removal of residual solvent and water, the resulting film contains imprinted porous cavities virtually throughout the surface of the film. Self-assembly of water droplets and their self-removal strategy (by drying) makes the breath figure process more promising and attractive technique in producing micro-structured materials.

We had also shown that the addition of small amount of PEG (10 wt %) is necessary to obtain uniformly sized pores in the PLGA film. When there are no PEG molecules in the blend, the pores and the resulting hydrogel pattern through the breath figure PLGA (Figure 38A and 38B) obtained are highly asymmetric in their arrangement. As it can be seen from Figure 38A, the PLGA film is filled with pores throughout the surface, but most of the pores are closely packed to each other. Consequently, the pattern
formation on the hydrogels is in a similar fashion (Figure 38B). When adding 10% PEG
in the PLGA blend, the presence of hydrophilic PEG polymer acts like surfactant
molecules and self-assembles at the interface of the condensing water droplets and the
evaporating polymer solution. This leads to the regular arrangement of the water droplets
and hence, the formation of spherical pores in the PEG/PLGA breath figures. However,
increase in the quantity of PEG (> 10%) into the PLGA showed the formation of an
irregular patch of pores on the film surface (data not shown). This suggests that increase
in the amount of PEG strongly influences the condensed water droplets to spread and
coalesce with each other and thus, restrict the formation of porous cavities. Henceforth,
all other experiments and characterization were performed using the breath figure
PEG/PLGA template to produce highly ordered patterns on the hydrogel, unless indicated
otherwise.

Figure 38. Scanning electron microscopy images of breath figure PLGA film and the
corresponding PHEMA pattern from the PLGA film.
Figure 39A and 39B show the surface topography and cross-sectional images of a spin coated breath figure PEG/PLGA film as characterized by scanning electron microscopy (SEM). The film surface has monodisperse pores (honeycomb-like structure) with the average dimensions of 1.5 microns. The narrow size range distribution of these breath figure pores was observed over the larger area of the film (24 mm glass coverslip substrate) with little or no defects. By controlling the process parameters (relative humidity, temperature, concentration and volume of polymer solution), the monodisperse pores can be produced in a consistent manner. The inset to Figure 39A shows the high magnification image which indicates the pores are formed at least 1 µm apart from each other.

We hypothesized that the water-borne materials such as hydrogels can be easily cast on top of the breath figure template and polymerized to harden the material in-situ similarly to the PDMS curing process\textsuperscript{138}. In a soft lithography process, PDMS elastomers have been used to replicate the patterns from the breath figure polymer films\textsuperscript{139-141}. This process is based on mixing the PDMS monomer and the curing agent at a weight ratio of 10:1. Then, The PDMS solution is cured as it infiltrates into the pores of the breath figures. The cured PDMS is then peeled off from the breath figure films without destroying the breath figure template. However, when we used hydrogels to transfer the pattern, it was difficult to peel off the hydrogel pattern from the breath figure PEG/PLGA template without destroying the pattern. To avoid that, we simply soaked the entire sample (the hydrogel and PEG/PLGA composite) into dichloromethane for a minute which led to the immediate dissolution of the PEG/PLGA film. We did not observe any swelling or blemishes in the patterned hydrogel when it was soaked in dichloromethane.
Figure 39. Scanning electron microscopy images of breath figure PEG/PLGA template and patterned hydrogel using breath figure film as reverse template

A) Surface topography of breath figure PEG/PLGA film. The pore dimension is ~ 1.5 microns (Inset: High magnification view)
B) Cross-sectional view of breath figure PEG/PLGA film. (Inset: High magnification view)
C) Surface view of micron size protrusions on hydrogel surface formed from breath figure PEG/PLGA film. The dimension is ~ 1.5 microns which is comparable to the size of breath figure pores. The bead-like pattern on the surface is interconnected to each other which is the replicate of the interconnected breath figure pores. (Inset: High magnification)
D) and (E) depicts the cross-sectional images of patterned hydrogels showing the height of protrusions equivalent to the pore depth. (Inset: High magnification)
E) Tilted surface view of hydrogel showing the regular arrangement of bead-like pattern.
As described in the experimental section, a homogeneous precursor solution was prepared by dissolving the monomer (HEMA), cross-linker (MBA), TEMED and APS in distilled water. The PHEMA is polymerized through the chemical cross-linking of MBA to the monomer through the free radical polymerization induced by APS and TEMED. Upon casting, the hydrogel precursors wet the PEG/PLGA template (relatively hydrophilic) and spread uniformly over the porous film. The solution then penetrates into the porous cavities to form a hardened and transparent PHEMA hydrogel film. In the figure 39C, the surface features of the patterned PHEMA hydrogel is shown. Regular arrays of solid protrusions (bead-like structures) are observed on the surface, once the porous PEG/PLGA is sacrificed by dissolving in dichloromethane. The size of the individual protrusions on the hydrogel surface is of the same order as the pore size of the PEG/PLGA film. As seen from the inset to figure 39C, the beads are formed separated from each other. From the cross-sectional SEM images shown in Figures 39D and 39E, the patterns formed on the surface are a monolayer with high fidelity. The inset to the figure 39E confirms that the height of the bead-like structure is equivalent to the pore depth (~ 1 µm). Figure 39F shows a side-angle view of the surface with the regular arrangement of spherically shaped protrusions on the hydrogel surface.

Further analysis was performed using confocal microscopy to confirm that the precursor solution is drawn into the pores by the wetting of the surface and the capillary action. Figure 40A shows the schematic illustration of casting of the hydrogel precursors containing fluorescein sodium salt (20 µg/mL) onto the PEG/PLGA template. The confocal image shown in Figure 40B represents a xy scan of the whole sample (PEG/PLGA template on the top and the hydrogel at the bottom) excited at 488 nm using
green filters. The image was captured without dissolving the PEG/PLGA template and shows the presence of fluorescein compound embedded into the spherical beads of the hydrogel pattern occupied in the voids of the breath figures. Additionally, the same sample was freeze-fractured in the cryogenic SEM chamber to view the cross-sectional feature of the whole sample after coating with a thin gold layer. As shown in Figures 40C (low magnification) and 40D (high magnification), both the breath figure template (at the bottom) and the hydrogel (at the top) can be seen from the differences in the contrast of the images. Arrows (shown in Figures 40C and 40D) represent the infiltration of precursors into the pores. It is important that the precursors should have low viscosity prior to casting. When the free radical initiator (APS solution) is added into the precursors (last step in the preparation of precursors), the precursors begin to polymerize instantly and increases the viscosity of the solution. We observed that the increase in viscosity prevents uniform spreading of the precursor solution across the PEG/PLGA template and hence, results into disordered patterns on the hydrogels.
Figure 40. Confocal and SEM analysis of whole sample (hydrogel and PEG/PLGA template)
A) Illustrates the loading of hydrogel precursors dissolved with fluorescein salt.
B) Confocal image of confirming the bead-like structures into which the fluorescein salt embedded into the hydrogel.
C) and D) shows low and high magnification of cross-section view of both hydrogel and breath figure PEG/PLGA film. From the differences in the contrast of the images, it is clearly seen the infiltration of hydrogel precursors into the breath figures.
We then arranged the two breath figure films for micro-patterning on both sides of the hydrogel thin film. For that, we placed the precursor solution between two breath figure PEG/PLGA films. Due to the capillary action, the hydrogel precursors are drawn upward and downward simultaneously to fill up the pores of the breath figure films. Similar to the single side patterning, the PEG/PLGA templates were again dissolved in dichloromethane to leave the bead-like structures on both sides of the PHEMA. Figure 41A shows the schematic illustration of sandwiching the hydrogel precursors between two breath figures and the resulting micro-patterned hydrogel. As shown in Figure 41B and 41C, the microbeads protruding on the surface are clearly visible on both sides of the hydrogel as assessed by cross-sectional SEM. From the cross-sectional view, the thickness of the patterned hydrogel measured is around 30 µm and it can be controlled by the volume of the precursors used.
Figure 41 shows the schematic diagram and SEM of double-side patterned PHEMA hydrogels.

A) Scheme illustrates the formation of double side patterned hydrogels. The hydrogel precursor solution is sandwiched between two breath figure PEG/PLGA films

B) Cross-sectional view of double side patterned hydrogels after removing templates.
Figure 42 shows the schematic illustration (A) of filling iron nanoparticles into the breath figures and placing of iron nanoparticles at the protrusion of hydrogel. B) shows the SEM images of breath figure PEG/PLGA template filled with Fe nanoparticles uniformly. C) shows the iron-filled free standing PEG/PLGA film and D) shows the magnet holding the film. E) and F) show the iron decorated onto the protrusion of the PHEMA hydrogel obtained by reverse template of breath figures.
We further explored the versatility of this simple casting technique by incorporating iron nanoparticles into the breath figure PEG/PLGA film. The filling of iron nanoparticles into the breath figure pores is depicted in Figure 42A. 0.1% RNIP (Reactive Nanoscale iron particles) homogeneous solution was taken in a beaker containing the breath figure PEG/PLGA film and the beaker was placed in the bath sonicator for 5-10 minutes. Prior to bath-sonication, RNIP solution was uniformly dispersed using the probe sonicator for 15-20 minutes. The utilization of bath-sonication has dual effects in the filling of iron nanoparticles into the breath figure pores. Firstly, it keeps the nanoparticles in suspension preventing their aggregation on the polymer surface. Secondly, it influences the infiltration of nanoparticles into the pores uniformly. As shown in Figure 42B, the iron particles are uniformly filled into the breath figure pores. We also noted that extensive washing of the film is required to remove the iron nanoparticles bound on the surface. However, the rinsing step did not remove the large amounts of the iron nanoparticles from the pores. This is because of the fact that the iron nanoparticles are drawn into the breath figure pores, aggregates into larger sizes and get locked into the pores firmly. The aggregation is clearly seen in the high resolution SEM shown in the inset to Figure 42B. The nanoparticles incorporated free standing PEG/PLGA film is shown in Figure 42C and the resulting film responded magnetically (Figure 42D) when the external magnetic field is applied. Furthermore, the hydrogel precursors were cast and cured upon these iron nanoparticles incorporated PEG/PLGA films. Figures 42C and 42D show the low and high magnification images of the reverse patterned PHEMA hydrogels decorated with the iron particles precisely on the bead-like protrusions. It was also noted that while sacrificing the PEG/PLGA template in
dichloromethane, the iron nanoparticles did not disintegrate from the pattern. Instead, they are packed intact into the protrusions on the surface of the patterned hydrogel.

The micro-fabrication approach shown in this study is convenient, versatile and does not involve any harsh conditions such as temperature, UV irradiation to polymerize the precursors to form PHEMA hydrogel\(^{128,134}\). The hydrogel precursors are dissolved in distilled water (solvent medium) in which the polymerization occurs simply by free radical formation and propagation. Hence, this approach can be easily applied to a variety of hydrogels and polymeric systems involving water or polar solvents as a reacting medium and induce polymerization intrinsically in the porous cavities of the breath figures. This led us to attempt the possibility of creating patterns directly on water-soluble polymers without involving any polymerization. To prove that concept, we chose a variety of biopolymers that are commercially available and easily soluble in water. The polymers selected were chitosan (1% in acetic acid) (Sigma Aldrich), hydrophobically modified chitosan (1% in acetic acid), gelatin (5% in distilled water and heated to dissolve completely) (sigma Aldrich), carboxymethyl cellulose (1% in distilled water) (sigma Aldrich) and poly (vinyl alcohol) (4% in distilled water). We prepared these homogeneous polymer solutions and cast them upon the breath figure PEG/PLGA template. Then the solution was air dried at the room temperature for a day to form a film on top of the breath figure template. In a similar fashion to obtain the PHEMA pattern, we were able to dissolve away the PEG/PLGA template leaving behind the pattern onto the surface of these polymers. In Figure 43, low and high resolution (inset) SEM images shown for all the polymers chosen and it is apparent that these water soluble polymers are
able to penetrate into the breath figure pores and replicate the pattern on their surface with high fidelity.

**Figure 43** shows the micro-patterned biopolymers obtained using the reverse template. Scale bar indicates 10 µm and insets are high magnification images (Scale bar ~ 2 µm).
Thus, the micro-fabrication of hydrogels and biopolymers using breath figures facilitate a new route to place biomolecules (proteins, cells, antibodies, functionalized bio-nanomaterials etc.,) precisely in the ordered micro-patterned surfaces. For example, magnetic nanoparticles conjugated with biological molecules have several advantages in comparison to fluorescently labeled molecules. Fluorescent labeled detection decreases in the intensity because of photobleach over a period of time whereas biomolecules labeled magnetic nanoparticles are easily detectable with improved detectivity in the performance. Moreover, placing biomolecules or conjugated nanoparticles in the hydrogels provides an additional advantage, as the hydrogels are hydrophilic and strongly absorbs water into the matrix. They act like natural tissue mimicking in vivo function, thereby reduced denaturation of the biomolecules. Applications of these patterned hydrogels in cell culture substrates and micro-patterning of protein arrays will be detailed in subsequent publications.
6.5 Conclusions

In this study, a new breath figure templating approach was utilized to create micro-patterned PHEMA hydrogels. The breath figure based templating system is easy to perform and is a low-cost technique that can be scalable to a large volume. The SEM analysis showed that the bead-like patterns (average size ~1.5 µm) formed on the PHEMA surface are of the order of the same dimension of the original breath figure pores, which demonstrates the high fidelity in replicating the pattern. By incorporating iron nanoparticles into the pores, this technique was further extended to form micro-patterned hydrogels decorated with iron nanoparticles on the protrusions. The major attraction of breath figure casting approach lies in the versatility for patterning on a variety of soft materials, including water-soluble polymers. In summary, we propose a novel fabrication technique in the development of patterned topography on soft materials for applications in protein micropatterning arrays, cell culture substrates, tissue engineering and biosensors.
**GLOSSARY**

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PLGA</td>
<td>Poly (lactic-co-glycolic acid)</td>
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<tr>
<td>PEG</td>
<td>Poly (ethylene glycol)</td>
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<tr>
<td>GDD</td>
<td>Glaucoma Drainage Device</td>
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<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
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<tr>
<td>MMC</td>
<td>Mitomycin C</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>MCF-7</td>
<td>Breast cancer cell line (Michigan Cancer Foundation-7)</td>
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<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>DAPI</td>
<td>4’6-diamidino-2-phenylindole</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
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<tr>
<td>MBA</td>
<td><em>N,N</em>-Methylenebisacrylamide</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
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<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
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<tr>
<td>RNIP</td>
<td>Reactive Nanoscale Iron Particles</td>
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<tr>
<td>PHEMA</td>
<td>Poly (hydroxyethyl methacrylate)</td>
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Thiruselvam Ponnusamy was born in Thanjavur town in Tamil Nadu state of India. He attended Kalyanasundaram Higher Secondary School in Thanjavur. He pursued his Bachelor’s and Master’s degree in Chemical Technology specializing in Oils, Oleochemicals and Surfactants from University Institute of Chemical Technology (erstwhile UDCT), Mumbai. Before pursuing Master’s degree, he also worked as a techno-commercial engineer at Godrej Industries Limited (Vegoils Division) for two years (2003-2005).

He joined Tulane University, New Orleans, USA in 2007 to pursue the doctoral study in the department of Chemical and Biomolecular Engineering. His research towards the doctoral program was focused on the design and development of “breath figures” based biodegradable thin polymer films to deliver drugs in a controlled manner from medical device implants. He also received a distinguished graduate student award (2012) from the department of Chemical and Biomolecular engineering for his journal publication in Biomatter.