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Fabrication and Testing of Scaffolds for Cell Growth from Ionic Liquid Solubilized Fibroin

Maneesh Kumar Gupta
Wright State University

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FABRICATION AND TESTING OF SCAFFOLDS FOR CELL GROWTH FROM IONIC LIQUID SOLUBILIZED FIBROIN

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Engineering

BY

MANEESH KUMAR GUPTA
B.S. in Chemical Engineering, University of Illinois, 2004

2007
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Maneesh Kumar Gupta ENTITLED Fabrication and Testing of Scaffolds for Cell Growth from Ionic Liquid Solubilized Fibroin BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science in Engineering

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ABSTRACT


The advent of tissue engineering has spurred research into developing materials to act as scaffolds for tissue growth. These scaffolds require materials capable of mimicking the in vivo conditions so that tissues can be grown effectively in vitro. Silk is an attractive biomaterial for use in tissue engineering applications because of its slow degradation, excellent mechanical properties, and biocompatibility. The major objective of the research in this thesis is to demonstrate a simple method to cast films directly from silk fibroin dissolved in an ionic liquid. The films cast from the silk ionic liquid solution were found to support normal keratinocyte proliferation and differentiation. The versatility of the silk ionic liquid solutions and the ability to process large amounts of silk into materials with controlled surface topography directly from the dissolved silk ionic liquid solution make it an interesting material for a wide variety of tissue engineering applications.
## TABLE OF CONTENTS

I. INTRODUCTION .................................................. 1

II. BACKGROUND ..................................................... 3
   - Tissue Engineering ........................................... 3
   - Silk .......................................................... 4
     - Protein Structure ......................................... 4
     - Mechanical Properties .................................... 5
     - Processing .................................................. 6
     - Biocompatibility and Degradation ......................... 7
     - Tissue Engineering ......................................... 8
   - Ionic Liquids ................................................ 11

III. EXPERIMENTAL METHODS ....................................... 13
   - Fibroin Solution Preparation ................................ 13
   - Film Fabrication ............................................ 14
   - Film Characterization ....................................... 16
   - Cell Culture ................................................ 17
   - Keratinocyte Proliferation Study ............................ 17
   - Keratinocyte Differentiation Study ......................... 18
   - Cell Growth on Patterned Films .............................. 19
   - Image Analysis ............................................. 19
<table>
<thead>
<tr>
<th>IV. RESULTS AND DISCUSSION</th>
<th>. . . . . .</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Film Fabrication</td>
<td>. . . . . .</td>
<td>21</td>
</tr>
<tr>
<td>Characterization</td>
<td>. . . . . .</td>
<td>23</td>
</tr>
<tr>
<td>Keratinocyte Proliferation</td>
<td>. . . . . .</td>
<td>25</td>
</tr>
<tr>
<td>Keratinocyte Differentiation</td>
<td>. . . . . .</td>
<td>26</td>
</tr>
<tr>
<td>Keratinocyte Growth on Patterned Films</td>
<td>. . . . . .</td>
<td>29</td>
</tr>
<tr>
<td>V. CONCLUSION</td>
<td>. . . . . .</td>
<td>32</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>. . . . . .</td>
<td>34</td>
</tr>
</tbody>
</table>

APPENDICES

| A. Film Characterization Techniques              | . . . . . . | 38 |
| B. Image Analysis                                | . . . . . . | 40 |
| C. Patterns Fabricated in Silk Films             | . . . . . . | 42 |
| D. RT-PCR Data                                   | . . . . . . | 46 |
| E. Published Manuscript                          | . . . . . . | 49 |
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chemical Structures of Ionic Liquids</td>
<td>12</td>
</tr>
<tr>
<td>2.</td>
<td>Schematic for Fabrication of Patterned Silk Films Using PDMS Molds</td>
<td>15</td>
</tr>
<tr>
<td>3.</td>
<td>Schematic for Fabrication of Patterned Silk Films Using Metal Tem Grids</td>
<td>16</td>
</tr>
<tr>
<td>4.</td>
<td>Silk Film Imprinted With Pattern 1</td>
<td>22</td>
</tr>
<tr>
<td>5.</td>
<td>Silk Film Imprintesd with a 300 Mesh Hexagonal TEM Grid</td>
<td>22</td>
</tr>
<tr>
<td>6.</td>
<td>ATR-FTIR Spectra of Amide I Peak</td>
<td>24</td>
</tr>
<tr>
<td>7.</td>
<td>ATR-FTIR Spectra of BMIC Cl Peak at 1463 cm$^{-1}$</td>
<td>24</td>
</tr>
<tr>
<td>8.</td>
<td>Optical Micrographs of Keratinocytes.</td>
<td>25</td>
</tr>
<tr>
<td>9.</td>
<td>Keratinocyte Proliferation Cell Counts</td>
<td>26</td>
</tr>
<tr>
<td>10.</td>
<td>mRNA Transcript Levels of SERPIN B9</td>
<td>28</td>
</tr>
<tr>
<td>11.</td>
<td>mRNA Transcript Levels of PADI 3</td>
<td>28</td>
</tr>
<tr>
<td>13.</td>
<td>Histograms of Cell Angle Data</td>
<td>30</td>
</tr>
</tbody>
</table>

**Appendices**

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1.</td>
<td>Image Analysis of Cells Grown on Pattern 2</td>
<td>40</td>
</tr>
<tr>
<td>B2.</td>
<td>Image Analysis of Cells Grown on Normal Tissue Culture Plates</td>
<td>41</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>C1</td>
<td>Saw Tooth Pattern 1</td>
<td>42</td>
</tr>
<tr>
<td>C2</td>
<td>Saw Tooth Pattern 2</td>
<td>43</td>
</tr>
<tr>
<td>C3</td>
<td>Square Wave Pattern 1</td>
<td>44</td>
</tr>
<tr>
<td>C4</td>
<td>Square Wave Pattern 2</td>
<td>45</td>
</tr>
<tr>
<td>D1</td>
<td>RT-PCR Data Normal Tissue Culture Plates</td>
<td>46</td>
</tr>
<tr>
<td>D2</td>
<td>RT-PCR Data Silk Coated Tissue Culture Plates</td>
<td>47</td>
</tr>
<tr>
<td>D3</td>
<td>RT-PCR Data Collagen Coated Tissue Culture Plates</td>
<td>48</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mechanical Properties</td>
<td>5</td>
</tr>
<tr>
<td>2. Summary of Tissue Engineering Studies</td>
<td>9</td>
</tr>
</tbody>
</table>
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DEDICATION

To my mother and father, Santosh and Rakesh Gupta, for their love, encouragement, and understanding.
I. INTRODUCTION

Silk fibers isolated from cocoons of the silkworm *Bombyx Mori* have long been cultivated by humans for use in textiles. The process of rearing silkworms for the production of silk fibers, termed sericulture, has existed for thousands of years. Silk textiles have been valued historically for their extraordinary beauty, strength, and tactile appeal. The combination of these properties along with widespread availability and ease of cultivation have expanded the potential uses for silk fibers. Due to its excellent mechanical properties silk was used for light protective armor and even in the early production of bullet proof vests. The biocompatibility of silk made it an excellent choice for use as a biomaterial primarily in non-absorbable sutures. However, the emergence of synthetic fibers such as nylon displaced the utilization of silk in certain industries.

Recently, the emergence of tissue engineering has sparked considerable interest in silk as a scaffold material for tissue growth. The field of tissue engineering requires scaffold materials to allow for tissue growth *in vitro* before they are implanted *in vivo*. As such materials with a unique combination biocompatibility and mechanical strength are required. Recent studies have shown fabrication of scaffolds of various geometries (films, fibers, mats, and sponges) from silk fibroin protein solutions. The biocompatibility and mechanical strength of silk scaffolds along with the abundant availability of silk fibers have made silk an interesting material for tissue engineering applications. The aim of this work was to develop a new method for fabrication of
scaffolds for tissue growth from regenerated solutions of silk fibroin in ionic liquid.
Subsequently, these scaffolds were characterized and tested for their ability to support
adhesion, proliferation, and differentiation of human cells.
II. BACKGROUND

TISSUE ENGINEERING

In the past several years the emergence of tissue engineering has created an unprecedented demand for novel biomaterials with unique physical, chemical, and biological properties. These materials are required as scaffolds on which cells can be seeded and grown into various tissues before they are implanted in vivo. As such the material must be suitable for cell adhesion, growth, and differentiation. Furthermore, the material must provide significant mechanical stability, while also allowing room for the expansion of the tissue. Currently significant research is being carried out to develop potential biomaterials for various tissue engineering studies. One major challenge in developing suitable scaffolds is designing materials that allow for active adhesion and growth of cells while retaining the desired morphology and genetic expression.

In general cellular interactions with the substrate surface can be controlled via three distinct routes. First, it is well known that the substrate surface chemistry plays an exceedingly important role in the behavior of cells upon the surface. The addition of ligands or specific peptide sequences can allow for the specific binding of certain cell types or a distinct morphology of a particular cell type. Secondly, it is known that cells are able to actively probe and respond to changes in substrate mechanics. Finally, it has only recently become apparent that the surface topography of a scaffold can direct the
growth and adhesion of seeded cells and even impact the differentiation and genetic expression of those cells.\textsuperscript{4,5,6}

SILK

The fiber spun by the silkworm $B.\text{Mori}$ is composed almost entirely of the protein fibroin. The fibroin protein is produced in large quantities and stored in a pair of silk glands within the worm. During the spinning process a single continuous filament is extruded from each of the silk glands through a spinneret. The two filaments are cemented together to form a single fiber. The fibers are coated with glue-like proteins called sericin which hold the fibers together in the shape of the cocoon.\textsuperscript{7,8}

Protein Structure

The fibroin protein is actually a pair of proteins covalently linked through a disulfide bond. The heavier protein called the heavy chain is comprised of 5,263 amino acids with a total molecular weight of 391 kDa. The much smaller light chain protein only has a total molecular weight of 26 kDa. The heavy chain protein is divided into twelve highly crystalline regions separated by amorphous linker regions 42 to 43 amino acids in length. The amino acid composition of the heavy chain is as follows: 45.9% glycine (G), 30.3% alanine (A), 12.1% serine (S), 5.3% tyrosine (Y), 1.8% valine (V), and 4.7% of the remaining 15 amino acid residues. The crystalline regions are highly repetitive containing 2,377 repeats of the dipeptide G-X. In 64% of the repeats, residue X is alanine, 22% is serine, 10% is tyrosine, and 3% is valine. The dipeptide G-X is
commonly repeated to form two hexapeptides GAGAGS and GAGAGY which account for roughly 70% of the crystalline region.\textsuperscript{9}

**Mechanical Properties**

The abundance of the GAGAGS and GAGAGY motifs promote folding of the crystalline region of the protein into an anti-parallel $\beta$-pleated sheet secondary structure. This highly stabilized secondary structure is thought to account for the impressive mechanical properties of silk fibers. Table 1 provides a comparison of the mechanical properties of silk fibers with those of other common natural and synthetic polymers. Silk fibers possess a unique combination of strength and toughness.\textsuperscript{10-12} The combination of these properties is not typically found in other structural polymers. For example, collagen a biopolymer commonly used as a tissue engineering scaffold has greater elasticity than that of silk. However, the tensile strength of collagen is several orders of magnitude less than that of silk. Similarly, when compared with high performance synthetic fibers such as Kevlar, silk shows a much greater elasticity. Another unique mechanical property of silk is its ability to withstand compression. These mechanical properties along with the biocompatibility, slow degradation rate, and high yield of sericulture led silk to be widely employed as a suture material for many decades.\textsuperscript{10}

<table>
<thead>
<tr>
<th>Material</th>
<th>UTS (MPa)</th>
<th>Modulus (GPa)</th>
<th>% Strain at Break</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. Mori</em> silk (w/ sericin)</td>
<td>500</td>
<td>5-12</td>
<td>19</td>
</tr>
<tr>
<td><em>B. Mori</em> silk (w/o sericin)</td>
<td>610-690</td>
<td>15-17</td>
<td>4-16</td>
</tr>
<tr>
<td>Spider Silk</td>
<td>875-972</td>
<td>11-13</td>
<td>17-18</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.9-7.4</td>
<td>0.0018-0.0146</td>
<td>24-68</td>
</tr>
<tr>
<td>Bone</td>
<td>160</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>Kevlar (49 fiber)</td>
<td>3600</td>
<td>130</td>
<td>2.7</td>
</tr>
<tr>
<td>Synthetic Rubber</td>
<td>50</td>
<td>0.001</td>
<td>850</td>
</tr>
</tbody>
</table>
Processing

The process by which silk fibroin solutions are regenerated from silkworm cocoons generally consists of two primary activities. The first is the removal of sericin proteins and the isolation of pure fibroin fibers. The second step is the dissolution of these purified fibers in a solvent to generate a solution from which various scaffolds may be fabricated.\textsuperscript{13}

Traditional sericulture techniques collect and reel silk fibers by soaking cocoons in boiling water to soften the sericin proteins and free the silk fibers. However, this method is rarely used for purification of silk fibers intended for dissolution. In this case the most widely used protocol involves soaking silk cocoons in an aqueous solution of 0.05\% (w/v) Na\textsubscript{2}CO\textsubscript{3} for 60 minutes at 100 °C. A study conducted by Yamada \textit{et al} has shown that these and other sericin removal techniques often result in unintended degradation of fibroin proteins.\textsuperscript{13} The authors of the study suggest a sericin removal method involving soaking cocoons in a buffered aqueous solution of 8 M urea. Experiments performed by the authors determined that soaking cocoons in this solution at 80 °C for 30 minutes offered complete removal of sericin proteins with minimal degradation to the fibroin proteins.

Unfortunately, due to their highly stabilized secondary structure silk fibroin proteins have very limited solubility. Traditionally, strong ionic solvents such as 9.3 M LiBr were used to disrupt the extensive hydrogen bonding in the protein secondary structure. The use of such solvents requires extensive dialysis to yield aqueous solutions suitable for the fabrication of cellular scaffolds. Alternatively aqueous solutions of silk fibroin have been lyophilized resulting in a solid silk material with a disrupted crystalline
structure. This material can be redissolved in various organic solvents such as 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). While both aqueous and HFIP solutions of silk fibroin have been used to fabricate numerous scaffolds, each has its own limitations. Aqueous solutions tend to be unstable with a typical shelf life of one to two days. HFIP solutions on the other hand are extremely corrosive and toxic requiring considerable care in handling. Furthermore, preparation of each of these solutions requires multiple steps and is very labor intensive.

Previous work in our laboratory has shown the ability of silk fibroin to dissolve in di- and trialkylimidazolium based ionic liquids. Dissolution of silk into ionic liquids is a one step process in which fibers are mixed with the powdered ionic liquid and subsequently heated in order to dissolve the silk. Ionic liquid solutions of silk also hold other advantages over the traditional aqueous and HFIP solutions. Ionic liquid solutions of silk are stable for extended periods of time when compared to aqueous solutions. Ionic liquids are also known as “green” solvents due to their negligible vapor pressure and low toxicity. This allows ionic liquid solutions to be handled without specialized air handling or breathing apparatus. The high toxicity and volatility of HFIP solutions makes such breathing equipment necessary when working with them. A previous study from our laboratory has also shown the capability to fabricate fibers from ionic liquid solutions.

**Biocompatibility and Degradation**

The in vivo biocompatibility and degradation of silk have been extensively studied due to its abundant use as a suture material. Initially it had been thought that silk was responsible for immunological reactions in humans. However, it was later
established that this response is directly linked to presence of the sericin glue-like proteins on the implanted biomaterial. Studies have shown that the complete removal of sericin through various stripping processes allows for the fabrication of silk scaffolds with immunological responses similar to those of other widely used scaffold biomaterials.\textsuperscript{10,16,17}

According to definitions provided by the US Pharmacopoeia silk is not considered a degradable biomaterial. The literature, however, does show silk to be degradable over much longer time periods. It has been found that silk sutures lose most of their tensile strength one year after implantation \textit{in vivo} and are unidentifiable two years after implantation. It must be noted that numerous factors ranging from shape and size, implantation site, animal model, physiological health greatly affect the degradation rate of silk fibroin biomaterials \textit{in vivo}. A clear understanding of the relationship between these variables and the degradation rate has currently not been established. \textit{In vitro} it has been demonstrated that proteolytic enzymes such as chymotrypsin act to cleave the less crystalline linker regions of the fibroin molecule. It is thought that the remaining fragments of the fibroin protein are absorbed through endocytosis.\textsuperscript{10,16,17}

\textit{Tissue Engineering}

Numerous studies have fabricated cellular scaffolds in varying geometries from fibroin solutions and shown their ability to support the adhesion and proliferation of mammalian cell lines.\textsuperscript{18-32} Table 2 provides a summary of various types of fibroin scaffold geometries and cell lines that have been tested for tissue engineering applications. In general it is found that silk fibroin films quite readily support the
adhesion and proliferation of numerous cell lines and primary human cell types. These cells typically show normal morphology and behavior. The most common scaffold geometries tested have been films, mats, sponges, and fibers.

### TABLE 2
Summary of Tissue Engineering Studies

<table>
<thead>
<tr>
<th>Scaffold Geometry</th>
<th>Cell Line</th>
<th>Observations</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroin Film</td>
<td>L-929 mouse fibroblast</td>
<td>Comparable proliferation to collagen films</td>
<td>Minoura et al [18]</td>
</tr>
<tr>
<td>Fibroin Film</td>
<td>SE116 (human colon adenocarcinoma); KB (human mouth epidermoid carcinoma); Colo201 (human colon adenocarcinoma); QG56 (human lung carcinoma)</td>
<td>Comparable proliferation to collagen films as well as production rates of carcinoembryonic antigen (CEA)</td>
<td>Inouye et al [19]</td>
</tr>
<tr>
<td>Fibroin Film</td>
<td>Saos-2 (human osteoblast-like cells)</td>
<td>Apparent bone formation on fibroin films that was enhanced through RGD coupling</td>
<td>Sofial et al [20]</td>
</tr>
<tr>
<td>Fibroin Fibers</td>
<td>hBMSC (human bone marrow stromal cells); human adult anterior cruciate ligament</td>
<td>Supported ligament development in vivo</td>
<td>Altman et al [21]</td>
</tr>
<tr>
<td>Non-woven Fibroin Net</td>
<td>HDMEC (human dermal microvascular endothelial cells); HUVEC (human umbilical vein endothelial cells); HPMEC-ST1.6R (human pulmonary microvascular endothelial cells); ISO-HAS-1 (hemangioma)</td>
<td>Supported growth of human macro- and microvascular cells with normal structural and functional phenotypes</td>
<td>Unger et al [22]</td>
</tr>
<tr>
<td>Electrospun Fibroin Mat</td>
<td>hBMSC (human bone marrow stromal cells)</td>
<td>Supported adhesion and proliferation of cells</td>
<td>Jin et al [24]</td>
</tr>
<tr>
<td>Electrospun Fibroin Mat</td>
<td>NHOK (normal human oral keratinocytes); NHEK (normal human epidermal keratinocytes);</td>
<td>Supported adhesion and proliferation of cells and promoted spreading of type I collagen</td>
<td>Min et al [25]</td>
</tr>
<tr>
<td>Fibroin Fibers</td>
<td>hBMSC (human bone marrow stromal cells); human adult anterior cruciate ligament fibroblasts</td>
<td>RGD modification of fibers enhanced adhesion and proliferation of cells along with significant increase in spreading of type I collagen</td>
<td>Chen et al [26]</td>
</tr>
<tr>
<td>Fibroin Sponges</td>
<td>hMSC (human mesenchymal stem cells)</td>
<td>Supported growth of bone like morphologies not capable of being formed on collagen scaffolds</td>
<td>Meinel et al [27]</td>
</tr>
<tr>
<td>Fibroin Sponges</td>
<td>hMSC (human mesenchymal stem cells)</td>
<td>Supported chondrogenesis of hMSC similar to collagen with enhanced mechanical properties</td>
<td>Meinel et al [28]</td>
</tr>
<tr>
<td>Fibroin Films, Woven Fibroin Mats, and Non-woven Fibroin Mats</td>
<td>NHOK (normal human oral keratinocytes)</td>
<td>Fibroin mats showed greater spreading of type I collagen than films due to higher surface area</td>
<td>Min et al [29]</td>
</tr>
<tr>
<td>Fibroin Films</td>
<td>hMSC (human mesenchymal stem cells)</td>
<td>Films supported growth and proliferation of cells</td>
<td>Jin et al [30]</td>
</tr>
<tr>
<td>Fibroin Films</td>
<td>hMSC (human mesenchymal stem cells)</td>
<td>Films supported growth, proliferation, and differentiation of cells</td>
<td>Wang et al [31]</td>
</tr>
<tr>
<td>Electrospun Fibroin Mat</td>
<td>hMSC (human mesenchymal stem cells)</td>
<td>Supported mineralization and bone formation</td>
<td>Li et al [32]</td>
</tr>
</tbody>
</table>
Films are typically tested by coating tissue culture plates with fibroin protein solutions. Freestanding mats have been fabricated by making meshes of purified silk fiber. Mats with nano-scale fibers have been demonstrated by electrospinning fibroin solutions. Both fibroin films and mats have been shown to support adhesion and growth of numerous cell lines. Mats, however, have been shown to better support the formation of extracellular matrix proteins due to the greater surface area available for cellular interaction. Silk fibroin sponges have been made by mixing granular salts in HFIP solutions of silk. The mixture is then cast as a film and soaked in water to dissolve away the salt. Removal of the salt particles introduces porosity in the film that can be controlled via the granularity of the salt used. Sponges have been tested as scaffolds for growth of both bone and cartilage type tissues. Finally, purified fibroin fibers have been woven together and developed as scaffolds for growth of anterior cruciate ligament type tissues.

Comparison of silk scaffolds with collagen based scaffolds typically show similar adhesion and proliferation patterns on both. Certain studies have shown greater proliferation rates of cells grown on collagen matrices. Collagen proteins are known to contain the RGD (arginine-glycine-aspartate) peptide sequence which promotes binding of cells to the surface. This disparity has been remedied in many cases through cross linking RGD peptides to surfaces of silk scaffolds. The cross linking typically occurs through a covalent bond between the amino terminus of the peptide and activated carboxylate groups in the silk fibroin protein. Carboxylate groups are found on the side chains of aspartic and glutamic acid residues which occur quite regularly in the 42-43 amino acid linker regions of the heavy chain protein. Attachment of binding peptides to
silk fibroin matrices has been shown to enhance cellular proliferation similar to levels found on collagen matrices.

IONIC LIQUIDS

Ionic liquids are a class of low-melting organic salts. More specifically ionic liquids are those salts with melting points below 100 °C. These compounds have become increasingly interesting for applications in “green chemistry”. Due to their highly ionic nature these salts have negligible vapor pressure when compared to traditional organic solvents. This allows ionic liquids to be used without fear of releasing harmful vapors and the associated respiratory risks. However, ionic liquids are not non-toxic. They have been shown to pose a toxicity risk when exposed to skin or ingested.

Ionic liquids have presented a new avenue in biotechnology for enzyme catalysis. Ionic liquids are capable of dissolving enzymes without disrupting the secondary protein structure unlike most polar organic solvents. In fact many studies have shown ionic liquid solvents to stabilize enzymes such that reactions can take place at more extreme temperatures than previously possible in aqueous environments. The solubility of proteins in ionic liquids was expanded further with the dissolution of structural biomaterials like cellulose, keratin, and silk.

Previous work in our laboratory, has focused on dissolution of silk fibers in ionic liquids. It was found that the identity of both the cation and anion were crucial in determining the solubility of silk. Various anions were paired with the dialkylimidazolium cations 1-butyl-3methylimidazolium (BMIM) and 1-ethyl-3-methylimidazolium (EMIM) (Figure 1). The chloride anion was found to have highest
solubility of silk with 13.2% and 23.3% (w/w) for BMIM and EMIM respectively. These solutions were used along with a methanol coagulation bath for fabrication of monofilament silk fibers.\textsuperscript{14,15}

Figure 1. Chemical Structures of Ionic Liquids. (A) BMIM Cl and (B) EMIM Cl
III. EXPERIMENTAL METHODS

FIBROIN SOLUTION PREPARATION

Silk cocoons were obtained from *B. mori* silkworms raised on a diet of Silkworm Chow (Mullberry Farms, Fallbrook, CA). Upon completion of the cocoons the live pupae were extracted in order to avoid potential contamination or degradation of the fibroin protein.

Sericin proteins were removed from the fibers following the procedure outlined by Yamada *et al.*13 Silk cocoons were soaked at 3.3% (w/v) in a solution of 8 M Urea containing 40 mM Tris-SO$_4$, and 0.5 M β-mercaptoethanol. The solution was heated to 90 ºC for 1 hr in a water bath. The silk fibers were stirred regularly to ensure complete removal of sericin proteins. The silk fibers were then removed and dried by centrifugation at 4000 RPM in a 50 ml tube. The fibers were then washed extensively with ultrapure distilled water (18 MΩ·cm) and again dried by centrifugation. The washing procedure was repeated five times to ensure complete removal trace urea and sericin from the fibers. The fibers were then lyophilized for 48 to 72 hours for removal of any residual water.

Dried silk fibers were subsequently dissolved in 1-butyl-3-methylimidazolium chloride (BMIC) ionic liquid (io-li-tec GmbH and Co, Denzlingen, Germany). Dissolution was carried out by first manually separating and chopping silk fibers to improve mixing with the ionic liquid. The ionic liquid powder was then added to the silk
fibers in a dry N\textsubscript{2} environment. The mixture was then heated to 90 °C for 1 hour to completely melt the ionic liquid powder. The fiber and ionic liquid mixture was then mixed for 30 seconds in a speed mixer (FlackTek Inc., Landrum, SC) and returned to heating at 90 °C for 1 hr. The mixing and heating cycles were repeated four more times until a solution of light amber color was obtained. This procedure was used to mix silk ionic liquid solutions ranging from 1 to 10% (w/w).

The resulting solutions were typically extremely viscous and often solid at room temperature. In order to lower the viscosity and melting points of these solutions ultrapure distilled water (18 MΩ·cm) was added in amounts ranging from 5 to 25% by weight. In order to add water the silk ionic liquid solutions were heated to 90 °C. The water was added in small increments 1-5 wt% to ensure proper mixing of the solution. This is critical because excessive localized dilution may result in precipitation or gelling of the solution.

**FILM FABRICATION**

Films were fabricated from the silk ionic liquid solutions using a methanol coagulation bath. The solution used had the following composition 7.5% (w/w) silk fibroin, 25% (w/w) water, and 67.5% (w/w) BMIM Cl. The silk ionic liquid solution was spun coat on glass and silicon substrates. The substrate was then immersed in a coagulation bath for 10 minutes. Submersion of silk ionic liquid solution in methanol results in crystallization of the silk fibroin film and dispersion of the ionic liquid solvent. The substrates were then removed from the methanol bath and the films were allowed to air dry.
This process was extended to allow for the fabrication of freestanding films with patterned surface topography. The patterning of films was done by two methods. The first one utilized polydimethylsiloxane (PDMS) molds as substrates for casting silk films (Figure 2). In this method silk solutions were spin coated on the surface of a PDMS mold with the desired topography. The mold was then submerged in methanol for 10 minutes to crystallize the silk film. The silk films were carefully released from the PDMS mold resulting in freestanding films with patterned surface topography.

![Figure 2. Schematic for Fabrication of Patterned Silk Films Using PDMS Molds.](image)

(A) PDMS stamp of the desired design is cast. (B) The silk solution is spin coated onto the PDMS stamp. (C) The coated stamp is submerged in methanol bath to extract the ionic liquid solvent and causing the silk film to crystallize. (D) The crystallized silk film is peeled from the stamp.

The second method involved the use of metal masks layered on the top surface of the silk solution (Figure 3). In this case a thin layer of silk ionic liquid solution was spin coated on the surface of a glass substrate. Next a metal transmission electron microscopy (TEM) grid with the desired features was placed on top of the silk solution. The substrate
was then submerged in the coagulation bath. Upon crystallization of the film the TEM grid was peeled away leaving behind the patterned silk film.

![Figure 3. Schematic for Fabrication of Patterned Silk Films Using Metal TEM Grids.](image)

(A) The silk solution is spin coated on a glass substrate. (B) TEM grid is placed on top of the solution. (C) The substrate is submerged in methanol to crystallize the silk film. (D) The TEM grid is peeled off of the patterned film.

FILM CHARACTERIZATION

Films were characterized using both optical and scanning electron microscopy (SEM). The topographical profiles of patterned films and PDMS molds were measured using white light interferometry (Wyko NT1100). White light interferometry is a non-contact optical profiling technique that allows for rapid measurement of surface features. Please refer to Appendix A for further details on white light interferometry.

The secondary structure of silk fibroin films and solutions was measured using attenuated total reflection fourier transformed infrared spectroscopy (ATR-FTIR). The removal of ionic liquid from films was also monitored using ATR-FTIR spectroscopy. ATR-FTIR spectroscopy is a spectroscopic method allowing FTIR measurements to be
made on membrane surfaces. Please refer to Appendix A for information on white light interferometry.

CELL CULTURE

Primary human epidermal keratinocytes were obtained from Cascade Biologics (Portland, OR) and grown in EpiLife Media supplemented with EpiLife Defined Growth Supplement (Cascade Biologics). Cells were maintained in 10 cm tissue culture plates and stored in a 37 °C, 5% CO₂ humidified incubator. Growth media was replaced every 48 hours and cells were subcultured upon reaching 80% confluence using Trypsin/EDTA solution and Trypsin Neutralizer solution (Cascade Biologics).

For cell proliferation and differentiation studies, keratinocytes were grown on normal plastic, silk coated, and collagen coated (BD Biosciences) tissue culture plates. Silk coated plates were prepared by spreading 150 µl of silk ionic liquid solution (7.5% (w/w) silk fibroin, 25% (w/w) water, and 67.5% (w/w) BMIM Cl) on 6 cm tissue culture plates. Next the plates were rinsed two times with 5 ml of methanol for 10 minutes each time. The silk coated plates were rinsed twice with 5 ml of distilled water after which they were air dried overnight. Prior to use in tissue culture plates were sterilized by exposure under a UV lamp for 15 minutes. Before seeding the cells on plates coated with silk, the plates were rinsed one time each with phosphate buffered saline (PBS) and growth media.
KERATINOCYTE PROLIFERATION STUDY

For cell proliferation studies, cells were counted and seeded at constant density on standard plastic, silk coated, and collagen coated tissue culture plates. The plates were maintained as described above. At 6, 24, 48, and 72 hr time points after seeding, images of numerous random fields were captured for each condition. Cells were counted in each field as a comparison of the proliferation rates of keratinocytes grown on the different substrates.

KERATINOCYTE DIFFERENTIATION STUDY

For cell differentiation studies, upon reaching 80% confluence cells were treated with either 10 nM 1α, 25-dihydroxyvitamin D3 (VD3) (Sigma, St. Louis, MO), 100 nM VD3, or control ethanol. The differentiation of known keratinocyte markers was monitored by measuring total RNA levels. For RNA extractions, cells were lysed directly on the culture plate and mRNA was harvested using the RNAeasy method according to the manufacturer’s protocol (Qiagen, Valencia, CA). Reverse transcription of mRNA was performed using the TaqMan Reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis was performed in a 96 well micro titer plate format on an ABI Prism 7900 sequence detection system using TaqMan Universal mastermix and Assay on Demand reagents specific for human genes Serpin B9, PADI 3, KLK10, Klf 4, and Ivl (PE Applied Biosystems, Foster City, CA). Each well was monitored for fluorescent dye and signals were considered significant if the fluorescence intensity significantly exceeded the standard deviation of the basic
fluorescence, defined as the threshold cycle \((C_T)\). Relative mRNA quantitation was performed using the comparative \(\Delta\Delta C_t\) method.\(^{41}\)

CELL GROWTH ON PATTERNED FILMS

Patterned silk films were fabricated as mentioned above. For cell growth studies, two patterns were chosen for film fabrication. The first, Pattern 1, has a saw tooth pattern with a peak to peak spacing of 6.6 \(\mu\)m and depth of 200 nm (Figure 4). The second, Pattern 2, is a square wave pattern with channel width of 10 \(\mu\)m, peak to peak spacing of 30 \(\mu\)m, and pattern depth of 1.5 \(\mu\)m. Fabricated films were kept hydrated in methanol prior to usage in order to prevent shrinking of feature sizes. Before seeding the films were rinsed twice with sterile water and exposed to UV radiation for 15 minutes. UV exposure was not seen to alter the properties of the films. Films were then rinsed once each with PBS and growth media. Films were placed in 6 cm tissue culture plates containing 4 ml of growth media. Keratinocytes were then seeded in the tissue culture plates. After 24 hours the films were transferred to new tissue culture plates. Images of the films were taken at 6, 24, 48, and 72 hrs.

IMAGE ANALYSIS

To quantify the effects of surface topography on cell alignment image analysis was performed by Dr. Lawrence Drummy. In order to make the images suitable for analysis a number of filters were applied to isolate the cells from the patterned background. A Fast Fourier Transform (FFT) of the original image was used to select reflections corresponding to the periodic spacings in the film pattern. An inverse FFT
was then performed and subtracted from the original image. The image was then inverted and flattened, using a high pass filter, so that large scale variations in background grayscale were removed. Next, the image was thresholded, a gaussian blur was applied to remove noise, and the image was then thresholded again yielding the final black and white image. Images of controls were analyzed in the same manner with the exception of the FFT. NIH Image was used to calculate the FFTs. Adobe Photoshop along with Fovea Pro plug-ins was used for application of the various filters, particle counting, and analysis. See Appendix B for examples of image analysis.
IV. RESULTS AND DISCUSSION

The ability of ionic liquids to dissolve silk fibroin proteins has provided a unique opportunity for the fabrication of silk biomaterials not possible thru traditional methods. As mentioned in the introduction the main thrust of this research has been the fabrication, characterization, and testing of scaffolds for tissue engineering from silk ionic liquid solutions.

FILM FABRICATION

Optically clear and mechanically robust silk films with controlled surface topography were fabricated from silk ionic liquid solutions. Films were cast in most cases from a 7.5% (w/w) silk fibroin, 25% (w/w) water, and 67.5% (w/w) BMIM Cl solution. Using this solution it was possible to cast films with a dried bulk thickness ranging from 0.5 to 6 μm depending on the spin speed used.

Freestanding patterned films were made using both PDMS molds and metal masks as templates. Using PDMS molds a wide variety of patterned surfaces were imprinted in silk films. Figure 4 shows an SEM micrograph and white light interferometry of a film cast using a PDMS mold of Pattern 1. Additional patterned films are shown in Appendix C. The dimensions of the pattern on the released film matched those of the PDMS mold. This was confirmed by white light interferometry. The diffraction spacings of a green laser beam passed through both the released film and the
PDMS mold were also compared to verify transfer of the pattern. The thickness of the features, however, was observed to shrink 15-20% upon complete drying of films with 6.5 wt% fibroin concentration. More dilute solutions were found to collapse even more upon drying.

Figure 4. Silk Film Imprinted with Pattern 1. 
(A) SEM Micrograph and (B) White Light Interferometry

Figure 5. Silk Film Imprinted with a 300 Mesh Hexagonal TEM Grid. 
(A) Optical Micrograph and (B) White Light Interferometry
Metal masks in the form of TEM grids were also used to make numerous patterned films. Patterns ranging from channels to hexagonal grids were fabricated by this method. Figure 5 shows an optical micrograph and white light interferometry of a silk film patterned with a 300 mesh hexagonal TEM grid. The size of the area patterned by this technique was limited to the size of the TEM grids available.

CHARACTERIZATION

ATR-FTIR spectroscopy was used to measure the crystalline structure of silk films. In its native state silk fibroin has a highly stabilized \( \beta \)-pleated sheet secondary structure. This structure is thought to be responsible for the impressive mechanical properties of silk fibers. Dissolution of silk fibers results in the disruption of the secondary structure to a random coil conformation.\textsuperscript{14,15} The fabrication of scaffolds with good mechanical properties requires the fibroin protein to return to its crystalline structure. Thus changes in the crystalline structure of silk fibroin between solution and film were monitored. Figure 6 shows reflectance FTIR spectra collected for silk ionic liquid solutions (solid black line) and silk films (dashed blue line) from 1600 to 1700 cm\(^{-1}\) wavenumbers. The shift in the amide I peak from ~ 1650 cm\(^{-1}\) to 1622 cm\(^{-1}\) wavenumbers is indicative of a transition from random coiled to \( \beta \)-pleated sheet secondary structure.\textsuperscript{42} This result suggests the methanol treatment used was successful in restoring the crystallinity of the silk fibroin.

Although ionic liquids have been termed “green” solvents numerous studies have shown toxicity.\textsuperscript{43,44} The application of ionic liquid solvents for fabrication of tissue engineering scaffolds would require the complete removal of ionic liquids in the
final biomaterial. ATR-FTIR spectroscopy was used to measure the presence of BMIM Cl in silk fibroin films. BMIM Cl has a characteristic IR peak at 1463 cm\(^{-1}\).\(^{15}\) The loss of this peak upon methanol treatment indicates complete removal of BMIM Cl from the silk film (Figure 7 solid black line indicates silk ionic liquid solution and the dashed blue line indicates silk film).\(^{15}\)
KERATIONOCYTE PROLIFERATION

The ability of silk fibroin films cast from ionic liquids to support the adhesion and proliferation of primary human cells was tested with normal human epidermal keratinocytes. In this study keratinocytes were seeded at equal density on standard tissue culture plates, collagen coated plates, and silk coated plates. Cells were counted on each substrate at 6, 24, 48, and 72 hours and plotted to compare the proliferation patterns of keratinocytes grown on each material.

Keratinocytes were found to adhere well to silk coated plates. Cells grown on silk plates (Figure 8A) showed similar morphology to those grown normal (Figure 8B) and collagen coated (Figure 8C) plates. The proliferation patterns of keratinocytes grown on all three substrates were also found to be similar (Figure 9). However, the growth of keratinocytes on collagen coated plates was found to be slightly higher at the 48 and 72 hour time points.

Figure 8. Optical Micrographs of Keratinocytes.
(A) Silk Coated, (B) Normal, and (C) Collagen Coated Tissue Culture Plates 24 hr After Seeding (100x Magnification)
Higher proliferation rates of cells grown on collagen compared with those on silk scaffolds have been previously reported in the literature. This is not surprising since collagen is a naturally occurring extracellular matrix protein with abundant RGD binding sites. Silk scaffolds have been shown to have similarly enhanced proliferation when decorated with RGD peptides. While, collagen scaffolds did have higher proliferation rates, the results of this study suggest that the use of ionic liquids solvents had no deleterious effects on the ability of silk scaffolds to support keratinocyte adhesion and growth as compared to standard tissue culture plates.

KERATIONOCYTE DIFFERENTIATION

In order to allow for the generation of specialized tissues in vitro, tissue engineering scaffolds must be capable of supporting the appropriate differentiation pathways. Though, silk films cast from ionic liquid were capable of supporting adhesion
and proliferation of keratinocytes, differentiation of cells is highly dependent upon the cellular environment. Thus the ability of these films to support known differentiation pathways of keratinocytes was tested.

\( 1\alpha, 25\)-dihydroxyvitamin D3 (VD3), the active form of vitamin D3, is known to induce differentiation in primary keratinocytes. Consequently, VD3 and its analogs have shown great potential in the treatment of skin abnormalities involving faulty differentiation of keratinocytes. VD3 binds to the vitamin D receptor (VDR) initiating a signaling pathway that results in the induction of several genes involved in keratinocyte differentiation including peptidylarginine deiminase (PADI) and serine proteinase inhibitor (SERPIN B) gene family members.

Keratinocyte differentiation was tested by seeding equal numbers of cells on normal plastic, collagen coated, and silk coated tissue culture plates. Once cells reached 80% confluence, they were treated with doses of 10 nM VD3, 100 nm VD3, and control ethanol. Differentiation of the cells was monitored by measuring changes in mRNA transcripts levels for the genes Serpin B9 and PADI 3 using Taqman based reverse transcriptase PCR (Figures 10 and 11 respectively).

It was observed that the cells grown on silk films cast from ionic liquids exhibited normal gene expression of differentiation markers PADI 3 and SERPIN B9 when treated with VD3 compared to cells grown on normal plastic and collagen coated tissue culture plates. Increased expression of PADI 3 and Serpin B9 was induced after VD3 treatments on all growth substrates. The cells grown on the silk films exhibited similar expression profiles to those grown on standard tissue culture plates or on collagen coated tissue
culture plates. Additionally, the expression of several other markers was found to be normal in cells grown on silk films cast from ionic liquids (Appendix C).

Figure 10. mRNA Transcript Levels of Serpin B9.

Figure 11. mRNA Transcript Levels of PADI 3 in Keratinocytes.
KERATINOCYTE GROWTH ON PATTERNED FILMS

Surface topography such as waves, grooves, and pillars have been shown to dramatically impact the morphology and functional behavior of cells.\textsuperscript{2,4,5,6} For example, 5 \( \mu \)m post features generated on elastomeric molds were found to align myocytes and fibroblasts.\textsuperscript{4} Freestanding silk films imprinted with Pattern 1 and Pattern 2 were tested. The patterned films were able to support adhesion and proliferation of primary human keratinocytes.

It was observed that the primary keratinocytes adhered to the micropatterned silk films within a few hours of seeding and exhibited normal cell spreading. Cells grown on Pattern 1 displayed typical morphology and were not visibly affected by the surface topography. The cells grown on Pattern 2 were observed to adhere to the ridges of the grooves and tended to elongate in the direction of the pattern. The alignment of the primary keratinocytes with the pattern observed at the 6 hr and 24 hr time points is shown in Figure 12.

Alignment of cells with patterned features was quantified using image analysis of keratinocytes grown on silk films imprinted with Pattern 2 and were compared with keratinocytes grown on normal tissue culture plates. Figure 13 shows histograms of cell angle data for both Pattern 2 (A) and the control surface (B). The cell angle was measured as the angle between the longest axis of the cell and the vertical axis of the image.
Figure 12. Optical Micrographs of Keratinocytes on Patterned Films. (A) Silk Films Imprinted with Pattern 2; (B) Normal Tissue Culture Plates

Figure 13. Histograms of Cell Angle Data. (A) Silk Films Imprinted with Pattern 2; (B) Normal Tissue Culture Plates
Based on the data, it is evident that the cells orient and elongate along the direction of the pattern. This is seen as a large number of cells clustered around 0° or 180° to the vertical axis of the image (Figure 13A) compared with the random distribution on the control surface (Figure 13B). This behavior was retained at the 6 and 24 hour time points. After that the cells began to divide and spread often straddling across the features. This data provides preliminary evidence for the impact of patterned silk films in controlling cell behavior. Further studies must be conducted to understand the impact of various topographical features on the morphology and functionality of cells. This method for fabricating patterned films does provide a basis for future work.
VI. CONCLUSION

The data presented in this study suggests that silk films cast from ionic liquids are capable of supporting growth and differentiation of primary human keratinocytes. The use of ionic liquids as solvents was not seen to have any negative effects on cell viability or behavior. Ionic liquids have allowed the fabrication of freestanding silk films with controlled surface topography. Preliminary results suggest that the topography of patterned silk films impacts the alignment of keratinocytes.

Future studies will focus on examining in greater depth the effects of silk substrates on keratinocyte morphology and behavior. For the future development of this technology, it will be important to fully understand the role of silk fibroin protein on keratinocyte growth and differentiation. Long term experiments will be performed to determine how silk proteins are metabolized by the keratinocytes. It will also be critical to understand the process by which the extracellular matrix is developed especially in concert with the metabolization of the scaffold. The final set of experiments will optimize the silk scaffolds by determining which topographical patterns are optimal for directing keratinocyte growth.

Silk fibroin is extremely versatile as a biological scaffold. The excellent mechanical and biological properties of silk allow fabrication of scaffolds of various geometries capable of supporting cell growth. Silk fibroin can be further decorated with peptides to alter cell growth and behavior. Now with the use of ionic liquids it is possible
to fabricate films with controlled topography. This incredible versatility makes silk an attractive material for tissue engineering applications. The ability of the scaffolds in this study to support growth and differentiation of keratinocyte cells give this technology the potential to revolutionize the way skin wounds are treated.
REFERENCES


WHITE LIGHT INTERFEROMETRY

In this study white light interferometry was used to profile the surfaces of patterned silk films and PDMS molds. White light interferometry utilizes a broad band illumination source in contrast with the monochromatic sources used in classical techniques. The light source is collimated and passed thru a beam splitter. One beam of light is reflected off the surface of the sample, and the other is reflected off an internal reference mirror. The beams are then recombined and captured by a CCD camera. Due to interference in the beams areas of light and dark “fringes” are formed. The area of highest intensity in the fringe pattern indicates regions with zero optical path difference between the sample and the reference mirror. The profile of the surface is measured by scanning vertically and measuring the “fringe” intensity at each pixel in the CCD during the scan. The data captured is then translated to surface profile data using provided software.46

ATTENUATED TOTAL REFLECTANCE – FTIR

ATR-FTIR spectroscopy was used to measure chemical and crystalline properties of silk fibroin solutions and films. FTIR (fourier transformed infrared) spectroscopy is a technique that allows for analysis of functional groups in molecules. Traditional FTIR sampling techniques were used for measurements of solid powders, liquids and gasses. ATR-FTIR is a technique developed to allow measurements of other surfaces such as films and fibers. Traditional FTIR operates by transmission of an infrared light beam
through the sample of interest. ATR-FTIR is performed by bringing the surface of the sample in close contact with a high refractive index prism. The IR beam is transmitted into the prism and is internally reflected at the interface between the prism and sample. Though, the beam is reflected internally it does penetrate a short distance in the sample. This allows for the collection of transmission-like FTIR spectra.\textsuperscript{47}
Figure B1. Image Analysis of Cells Grown on Pattern 2.
Figure B2. Image Analysis of Cells Grown on Normal Tissue Culture Plates
Figure C1. Saw Tooth Pattern 1. Maximum Peak Height of 1.2 μm
Figure C2. Saw Tooth Pattern 2.
100 μm Peak to Peak Spacing and Pattern Depth of 1.2 μm
Figure C3. Square Wave Pattern 1.
200 μm Peak to Peak Spacing and Pattern Depth of 1 μm
Figure C4. Square Wave Pattern 2. 
200 μm Peak to Peak Spacing and Pattern Depth of 1 μm
Figure D1. RNA Expression Levels in Keratinocytes Grown on Normal Plastic Tissue Culture Plates.
Figure D2. RNA Expression Levels in Keratinocytes Grown on Silk Coated Tissue Culture Plates
Figure D3. RNA Expression Levels in Keratinocytes Grown on Collagen Coated Tissue Culture Plates
APPENDIX E
PUBLISHED MANUSCRIPT

Patterned Silk Films Cast from Ionic Liquid Solubilized Fibroin as Scaffolds for Cell Growth

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Silk is an attractive biomaterial for use in tissue engineering applications because of its slow degradation, excellent mechanical properties, and biocompatibility. In this report, we demonstrate a simple method to cast patterned films directly from silk fibroin dissolved in an ionic liquid. The films cast from the silk ionic liquid solution were found to support normal cell proliferation and differentiation. The versatility of the silk ionic liquid solutions and the ability to process large amounts of silk into materials with controlled surface topography directly from the dissolved silk ionic liquid solution could enhance the desirability of biomaterials such as silk for a variety of applications.

Introduction

Of late, a considerable amount of interest has developed regarding the use of patterned and textured biomaterial surfaces for applications in tissue engineering and drug delivery. Recent research has shown the importance of surface topography and texture in controlling the shape, orientation, and gene expression of adherent cells.1 Historically, silk fibers have been widely used in biomedical applications as a nature material for repairing wound injuries. The recent ability to fabricate silk-based materials of various geometries including films, sponges, mats, and fibers, from purified silk fibroin solution has resulted in the reemergence of silk as a biomaterial. Silk is an attractive tissue engineering scaffold because of its slow degradation, excellent mechanical properties, and biocompatibility.2 Numerous studies have proven the biocompatibility of silk substrates in supporting the adhesion and proliferation of mammalian cells.3

Silk fibroin protein, isolated from the cocoon fiber of the Bombyx mori silkworm, consists of a 391 kDa heavy chain and 26 kDa light chain covalently linked through a disulfide bond.4 The heavy chain portion of the protein is divided into twelve crystalline regions separated by amorphous regions 30–40 amino acids in length. The crystalline regions are primarily composed of the GAGAG amino acid motif which assumes a hydrogen-bonded antiparallel β-sheet structure in the native fiber. The highly crystalline secondary structure of silk is thought to be responsible for the excellent mechanical characteristics. Native fibers are coated in gels like sericin proteins which hold the fibers together in the shape of the cocoon.4

Unfortunately, due to its stabilized secondary protein structure, silk fibroin has limited solubility characteristics, resulting in significant limitations regarding the fabrication of tissue engineering scaffolds. Previous work in our laboratory has shown the ability to dissolve silk fibroin in di- and trialkylimidazolium-based ionic liquids and the use of the resulting solutions to fabricate silk films and fibers.5,6 This holds many advantages over conventional methods. Traditionally, ionic aqueous solutions such as 9.3 M LiBr have been used to dissolve silk fibers. These solutions require extensive dialysis, yielding aqueous silk fibroin solutions with a shelf life of 1–2 days. Alternatively, aqueous solutions of silk fibroin have been lyophilized and redisolved in organic solvents such as 1,1,1,3,3,3-hexafluoropropanol (HFIP). These solvents are typically extremely corrosive and toxic, requiring considerable care in handling. Ionic liquids, on the other hand, have long been recognized as “green” solvents7 and have been used in the dissolution of silk,3,8 cellulose,8 and keratin.9 The resulting solution has extended stability and ease of processing of biopolymers into fibers and films.5,6,10 In this work, we have extended the versatility of the silk ionic liquid solutions and demonstrate the ability to fabricate silk fibroin films with controlled surface topography directly from the dissolved silk ionic liquid solution as scaffolds for cell growth.


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Figure 1. Schematic illustration of fabricating patterned silk films. (A) PDMS stamp of the desired design is cast. (B) The silk solution is spin-coated onto the PDMS stamp. (C) The coated stamp is submerged in a methanol bath to extract the ionic liquid solvent, causing the silk film to crystallize. (D) The crystallized silk film is peeled from the stamp.

Experimental Section

Fibroin Solution Preparation. Silk was obtained from B. mori silkworms raised on a diet of silkworm worm (Mulberry Farms, Fullbrook, CA). Live pupae were extracted from the cocoons prior to sericin removal in order to avoid potential contamination or degradation of the fibroin protein. Silk fibers were prepared as previously described. Briefly, silkworm cocoons were soaked at 3.3% (w/v) in a solution of 8 M urea, 40 mM Tris-SO₄, and 0.5 M β-mercaptoethanol and heated to 90°C for 1 h. The silk fiber was then extensively washed with ultrapure distilled water (18 MΩ-cm) and dried overnight under vacuum. Dried silk fibers were subsequently dissolved in 1-butyl-3-methylimidazolium chloride (BMIC) ionic liquid (IoLiTec GmbH and Co., Denzlingen, Germany) to form a 10% (w/v) solution. Dissolution was accomplished by heating a mixture of the silk fibroin and BMIC powder to 90°C for 1 h in order to melt the ionic liquid. The fiber and ionic liquid mixture was then mixed for 30 s in a speed mixer (FiskiTel, Inc., Londrum, SC) and returned to heating at 90°C for 1 h. The mixing was repeated four times until a solution with uniform consistency was obtained. In order to lower the viscosity and melting point of the solution, 25% (w/v) water was added to the heated solution. Care was taken to incrementally add the water (5% w/v increments) to the solution so as to avoid excessive localized dissolution and potential precipitation of silk from the solution. The final composition of the solution is 7.5% (w/v) silk fibroin, 25% (w/v) water, and 67.5% (w/v) BMIC.

Patterned Film Fabrication. Films were cast from the silkworm liquid solution using a methanol rinse bath. When submerged in methanol, the ionic liquid is extracted out of the silk solution into the methanol bath, resulting in crystallization of the silk fibroin protein into the antiparallel β-sheet secondary structure. Polydimethylsiloxane (PDMS) stamps with channels fabricated from patterned masters were used as micromolds for creating patterned silk films. The silk solution was spin-coated on top of the PDMS mold and submerged in methanol for 10 min, resulting in crystallization of the silk film. The optically clear silk film can then be released from the PDMS mold (Figure 1). Alternatively, TEM grids were used as molds to pattern the silk film. Here, the TEM grid is placed on top of the silk solution layered over a glass slide or silicon wafer. After rinsing with methanol, the TEM grid is lifted off, leaving behind a patterned film.

Film Characterization. Films were characterized using optical and scanning electron microscopy. Profiles of patterned films were measured using white light interferometry. Secondary structure and ionic liquid removal were monitored using attenuated total reflection Fourier transformed infrared spectroscopy (ATR-FTIR).

Cell Culture. Primary keratinocytes were purchased from Cascade Biologics (Portland, OR) and maintained in EpiLife media supplemented with EpiLife defined growth supplement (Cascade Biologies). For cell proliferation and differentiation assays, keratinocytes were grown on normal plastic, silk-coated, and collagen-coated (BD Biosciences) tissue culture plates. Silk-coated plates were prepared by evenly coating 6-cm tissue culture plates with 150 μL of silk ionic liquid solution. The plates were then rinsed twice with 5 mL of methanol for 10 min each time. Silk-coated plates were then rinsed twice with 5 mL of distilled water, after which they are dried and exposed under UV for 15 min. Before seeding cells, plates are rinsed once each with PBS and growth media. For cell proliferation studies, cells were seeded and counted at 6, 24, 48, and 72 h time points.

Cell Differentiation. For cell differentiation studies, upon reaching 80% confluence cells were treated with 10,25-dihydroxyvitamin D3 (VD3) (Sigma, St. Louis, MO). Differentiation was monitored by measuring mRNA transcript levels of known differentiation markers. For RNA studies, cells were lysed directly on the culture plate using the RNAasy method as per manufacturer’s protocol (Qiagen, Valencia, CA). Reverse transcription of RNA was performed using the TaqMan Reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR analysis was performed in a 96-well microtiter plate format on an ABI Prism 7000 sequence detection system using TaqMan Universal Mastermix and Assays on Demand reagents specific for human gene Serpin B9 and PADI III (PE Applied Biosystems, Foster City, CA). Each well was monitored for fluorescence dye, and signals were considered significant if the fluorescence intensity significantly exceeded the standard deviation of the basic fluorescence, defined as the threshold cycle (Ct). Relative mRNA quantification was performed using the comparative ΔΔCT method.

Image Analysis. In order to threshold the image and isolate the cells from the background, a number of filters were applied. As shown in the Supporting Information, the patterned silk background was removed by performing a fast Fourier transform (FFT) on the original image, selecting the reflections corresponding to the periodic spacings in the film, performing an inverse FFT, and subtracting the inverse FFT from the original image. The image was then inverted and flattened, using a high-pass filter, so that large-scale variations in background grayscale were removed. The image was then thresholded, a Gaussian blur was applied to remove noise, and the image was then thresholded again to produce the final black and white image of the cells (Figure S1). NIH Image was used for calculation of the FFTs, and the Adobe Photoshop software package with Fovea Pro plug-ins was used for application of the various filters as well as for particle counting and analysis. The data were then graphically represented as the angle of the cell with respect to the vertical axis of the image.

Results and Discussion

We have previously shown that silk fibroin can be dissolved in ionic liquids (ILs) such as 1-butyl-3-methylimidazolium chloride (BMIC) to form a stable silk fibroin solution. The solubility of silkworm silk fibroin in ILs is attributed to the ability of anion (Cl-) to disrupt the hydrogen bonding in the silk fibroin, and thus ILs are excellent solvents in which to dissolve silk. The silk liquid oligomer (SIL) solution obtained is clear and viscous. The viscosity of the solution can be decreased by adding water to the SIL solution. In most experiments, 7% (w/v) SIL solution was used to generate optically clear and mechanically robust silk films. In order to imprint features on silk films, elastomeric molds or metal masks were used as templates to generate surface features. The SIL solution is spin-coated directly onto a PDMS mold, or a metal mask can be placed on substrate coated with the SIL solution. We have previously demonstrated that acetonitrile or methanol can be used to extract out the IL.
Figure 2. Patterned silk films. SEM micrographs of (A) PDMS stamp patterned silk film with a peak-to-peak periodicity of 6.6 μm and (B) hexagonal patterns obtained using a TEM grid as a mask. (C) White light interferometry scan of hexagonal patterned silk film. (D) ATR-FTIR spectra of amide I region of silk (I) and BMIC (II) before (solid line) and after (dashed line) methanol treatment.

as well as cause the silk to crystallize. The resulting crystallized film is optically clear and mechanically robust, and can be released from the substrate after methanol treatment. The patterned films were analyzed by scanning electron microscopy (SEM). Figure 2A,B shows the surface features on the silk films generated using PDMS or TEM grid as molds, respectively. Numerous patterns with varying feature sizes were generated using both PDMS and TEM patterning techniques. A PDMS mold in conjunction with a metal mask can also be used in patterning both the top and bottom surfaces of a film. The features obtained on the silk films were well-defined and retained the features of the mask or mold used. We used white light interferometry in order to characterize the surface features of the patterned silk films (Figure 2C). The size features imprinted on the silk film were found to correspond to the size features of the mold with 15–20% decrease in the overall height when the films are allowed to dry for extended periods.

While ionic liquids have been considered “green solvents”, they are known to have toxic effects when in contact with biological systems. As such, the removal of ionic liquid is critical in the fabrication of silk films for cell growth experiments. Methanol can be used to extract out ILs from SIL-cast films, resulting in a transparent film, and the removal of ILs can be monitored using attenuated total reflectance FTIR (ATR-FTIR) spectroscopy. BMIC has a characteristic peak at 1463 cm⁻¹, and the loss of this peak upon methanol treatment indicates removal of BMIC from the silk film (Figure 2D, graph II). Methanol treatment also results in a conformational change in the secondary structure of the silk fibroin protein from random coil to β-sheet; this renders the silk film water-insoluble. This transition was also confirmed via FTIR spectroscopy (Figure 2D, graph I). This transition in secondary structure is indicated by the shift in the amide I peak from ~1650–1622 cm⁻¹ wavenumbers.

In addition to the mechanical stability, biocompatibility, and biodegradability of cell scaffolds, adequate cell adhesion and proliferation is also essential (see ref 17 for review). Since the treatment with methanol results in the removal of BMIC and crystallization of the silk film, we next tested the patterned silk films for their ability to support cell growth. Tissue culture plates were first coated with the SIL solution, treated with methanol, rinsed extensively in distilled water and sterilized by a brief exposure to UV radiation. Then, equal numbers of primary human epithelial keratinocytes were seeded onto the tissue culture dish coated with the silk film as well as onto bare plastic and collagen-coated tissue culture dishes for comparison. After allowing the cells to attach, we monitored cell growth by counting the number of cells that attached to the tissue culture plate and exhibiting normal cell morphology at indicated time points (Figure 3). The cells were found to adhere to tissue culture plates coated with silk, similar to the control and collagen-coated plate. In all cases, the cells on the different substrates exhibited similar cell morphology (Figure 3A,B).

Furthermore, no significant differences were observed in cell proliferation under the three different conditions (Figure 3C).

though a slight increase in cell proliferation was observed on cells grown on collagen, which is not surprising, since collagen is widely used as an extracellular matrix (ECM) for cell growth; however, it lacks the inherent mechanical properties of silk. Nonetheless, our results indicate that the silk film cast from the SIL solution is able to serve as a cell scaffold and allows for normal cell adhesion and proliferation.

We tested the effect of differentiation on gene expression in cells grown on silk films. 1α,25-Dihydroxyvitamin D3 (VD3), the hormonally active form of vitamin D3, has been shown to induce differentiation in primary keratinocytes. As a result, VD3 and its analogs show great promise as therapeutics in skin abnormalities. VD3 induces the vitamin D receptor (VDR) signaling pathway that results in the induction of several genes involved in cellular differentiation. We examined whether keratinocytes grown on the silk films generated from the SIL solution were able to undergo normal differentiation behavior upon exposure to VD3 by monitoring the expression of the differentiation markers. The expression of peptidylarginine deiminase (PADI), kallikrein (KLK), and serine proteinase inhibitor (SERPIN B) gene family members in primary normal human epidermal keratinocytes have been shown to be vitamin D dependent and are induced when cells undergo differentiation. The expression of PADI 3, Serpin B9, and KLK 10 in keratinocytes was analyzed by real-time RT-PCR (Figure 3D). We observed that the cells grown on patterned silk films cast from ionic liquids exhibited a normal gene expression pattern when treated with VD3; expression of PADI 3, Serpin B9, and KLK 10 was induced after VD3 treatment in all growth conditions. The cells grown on the silk films exhibited similar expression levels to those grown on standard tissue culture plates or on collagen-coated


Figure 3. (A, B) Optical micrographs of keratinocytes growing on silk or collagen coated tissue culture Petri dishes after 24 h of incubation (100× magnification). (C) Proliferation of keratinocytes on bare (control), collagen-coated, or silk-coated tissue culture Petri dishes. Cell count is represented as the number of cells/field of view. (D) Real-time RT-PCR showing the expression of known differentiation markers PADI 3, Serpin B9, and KLK10 in keratinocytes grown on standard tissue culture plates (control), silk-coated, and collagen-coated film tissue culture plates after 6 h exposure to VD3. The y-axis is the relative fold change in expression compared to untreated cells.

Figure 4. (A) Optical micrographs of keratinocytes growing on patterned silk films at 6 and 24 h. (B) Optical micrographs of cells growing on control (collagen-coated) tissue culture plates. Histograms displaying cell alignment on (C) patterned films and (D) control substrates (collagen-coated). The x-axis represents cell angle (measured as the angle between the longest axis of the cell and the vertical axis of the image), and the y-axis is cell count. The data show alignment of cells on patterned films as compared to the control.
tissue culture plates. In addition, expression of several other markers was found to be normal in cells grown on silk films cast from ionic liquids (data not shown).

Surface topography such as wavy features, grooves, and pillars has been shown to impact cell behavior. For example, 6 μm wave features generated on an elastomeric mold were found to align myoblasts. Silk films from the XIL solution patterned with periodic grooves with a spacing of 10 μm and 2 μm in depth were able to support the growth and differentiation of primary keratinocytes. We observed that the primary keratinocytes adhered to the micropatterned silk film within a few hours of seeding. The alignment of the primary keratinocytes with the pattern observed at the 6 h and 24 h time points is shown in Figure 4A.

Preliminary analysis using optical images of keratinocytes grown on the patterned and unpatterned silk films indicates that a significant number of cells grown on the patterned silk films exhibit preferential alignment along the surface pattern. This is demonstrated by the histogram showing the cell angle data for the patterned and unpatterned silk films (Figure 4C,D). The cell angle is the measured angle between the long axis of the cell and the vertical direction of the image (see Experimental Section). On the basis of the data, the cells appear to orient and elongate along the direction of the pattern, as seen by a large number of cells clustering around 0° or 180° to the vertical axis of the image (Figure 4C), and this behavior was retained until the cells begin to divide. In contrast, cells on the unpatterned films do not exhibit any preferential orientation (Figure 4D). We believe that our method of introducing surface features onto the silk films would allow us in the future to further study the effect of surface topography on cell behavior.

Together, the data suggests that silk films cast from the ionic liquid solution did not have any detrimental effect on cell viability, differentiation, or gene expression, and the use of ILs as a solvent for silk fibroin provides an avenue for the fabrication of silk scaffolds for tissue engineering applications. Our future studies will focus on the introduction of molecular signals by physical entrapment during the film processing step, coupling of small molecules onto the silk films after casting, and the study of various surface features in order to guide cellular behavior.

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Supporting Information Available: Additional experimental data as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.