ABSTRACT

MOHITI-ASLI, MAHSA. Controlled Release Systems Using Functional Nanofibers for Wound Healing and Tissue Engineering Applications. (Under the direction of Drs. Elizabeth G. Loboa and Behnam Pourdeyhimi.)

Controlled release systems are of great importance for the healthcare field, with specific emphasis on drug or compound delivery for wound healing and tissue engineering applications. Nonwoven structures are already used extensively in healthcare due to their large surface area, absorbent properties, facile processing scheme, and relative cost-effectiveness. The ability to create functional nonwoven structures with well-controlled morphologies and release properties can now be achieved on the nanoscale utilizing the electrospinning method. Electrospinning utilizes the interplay between electrical forces and surface tension to create fibers with submicron diameters, collected in random mats with high porosity, by applying a strong electric field between a charged drop of polymer solution and a collection plate. These nanofibrous mats provide a compliant mesh that not only resembles the natural extracellular matrix in vivo, but also provides a material with an extremely large surface area to volume ratio for maximizing the interaction of the carrier with a surrounding medium.

The purpose of this work was to develop functional nanofibers using electrospinning system and control the release rate of a variety of compounds from such structures as desired for multiple clinical applications. In all of our experiments polylactic acid (PLA) was used as the polymeric matrix and it was loaded with different compounds such as tricalcium phosphate (TCP) nanoparticles, as an osteoconductive compound for bone tissue engineering, silver nano particles, highly porous silver microparticles, and a silver nitrate base polymeric
solution as antibacterial, antimicrobial compound for wound healing application, and ibuprofen as an anti-inflammatory drug for wound healing applications. Fiber morphology, drug concentration and release medium temperature were the main parameters that we manipulated to control the release rate of drugs from nanofibrous structures. For all the drug loaded nanofibrous structures, we determined the release profile and the \textit{in vitro} cytotoxicity using human skin cells. For some of the drug/nanofiber composite structures we further pursued our experiments and evaluated their toxicity and functionality \textit{in vivo}.

Our finding confirmed that fiber morphology can change the release profile of drug from nanofibers and subsequently influence the activities of cells seeded on them. TCP nanoparticles encapsulated in porous fibers exhibited the highest release rate as compared to single component and core-sheath nanofibers. The differentiation of human adiposed drive stem cells (hASC) seeded on nanofibers was also influenced by fiber morphology and the highest differentiation observed for the cells seeded on porous fibers. Drug concentration also played an important role in determining the cytotoxicity of nanofibers coated with silver nitrate containing solution. Our results showed that higher silver content in nanofibers results in higher release rate as well as higher chances of cytotoxicity towards human skin cells. Same results observed when quantifying the release of ibuprofen from PLA nanofibers. Lastly, our preliminary \textit{in vivo} analysis using nude mice model for ibuprofen loaded nanofibers and pig model for silver containing nanofibers showed the potential of our developed functional nanofibers to be used in clinical applications.
Controlled Release Systems Using Functional Nanofibers for Wound Healing and Tissue Engineering Applications

by
Mahsa Mohiti-Asli

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Fiber and Polymer Science

Raleigh, North Carolina

2013

APPROVED BY:

__________________________________________  ______________________________________
Elizabeth G. Loboa                               Behnam Pourdeyhimi
Committee Co-Chair                                Committee Co-Chair

__________________________________________  ______________________________________
Saad Khan                                         Eunkyoung Shim
DEDICATION

To my supportive husband, my beloved parents and my encouraging brother.
BIOGRAPHY

Mahsa Mohiti-Asli was born on February 7, 1984 in Lahijan, Iran. She grew up in Karaj then attended Isfahan University of Technology to study textile engineering. In 2006, she graduated with her Bachelor of Science degree in textile engineering and started her Masters of Science at the Amirkabir University of Technology (Tehran Polytechnic). In 2009, she received the Masters of Science in textile engineering. Later in spring of 2010 she joined College of Textiles at NCSU to study Fiber and Polymer Science. Under the direction of Drs. Elizabeth Loboa and Behnam Pourdeyhimi, she researched on functional nanofibers for wound healing and tissue engineering application. She graduated with her Ph.D. in fiber and polymer science and a minor in Biomedical Engineering in August 2013. After completing the requirements of her degree she continued her research in Dr Loboa’s research group in Biomedical Engineering department as the postdoctoral research scholar.
ACKNOWLEDGMENTS

I would like to express my sincere gratitude and appreciation to Dr. Elizabeth Loboa and Dr. Behnam Pourdeyhimi who provided the opportunity to pursue my doctoral work. They are both great advisors and I would never forget their help, patience, and kindness. I would also like to acknowledge continues support of my advisory committee, Dr. Saad Khan and Dr. Eunkyoung Shim.

My Ph.D. research could not have been completed without help of other NCSU professors and technical staffs. My exceptional thanks go to Dr. Bernacki for her invaluable help in designing my experiments. I should also acknowledge Roberto Garcia, Chuck Mooney, and Dale Bachelor in Analytical Instrumentation Facility for their assistance.

I also greatly appreciate the Nonwovens Cooperative Research Center (NCRC) and the Chancellor’s Innovation fund (CIF) for funding my research.

I would like to extend my special thanks to my fellow graduate students in NCRC and all the members of Cell Mechanics Laboratory (CML) as well as my amazing friends who were always there for me.

Last but not the least, I would like to express my deepest appreciation to my family for their unconditional love, support, and encouragement throughout my entire education: my husband Mr. Shoayb Ziaei, my parents Mrs. Sareh Moazen and Mr. Abozar Mohiti-Asli and my brother Dr. Maziar Mohiti-Asli.
# TABLE OF CONTENTS

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

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

Chapter 1: Nanofibrous drug carriers for wound healing and tissue engineering applications: Modern strategies for controlled release ................................................................................................................................. 1

1.1 Introduction .......................................................................................................................................................... 2

1.2 Controlled release nanofibers for tissue engineering applications .......................................................... 4

1.3 Applications of controlled release nanofibers in wound healing .............................................................. 4

1.4 Release mechanisms .......................................................................................................................................... 7

1.5 Drug properties .................................................................................................................................................. 9

1.6 Nanofiber scaffolds ......................................................................................................................................... 11

1.7 Fiber morphology ........................................................................................................................................... 14

1.8 Thickness of scaffolds .................................................................................................................................... 18

1.9 New strategies applied for controlled release nanofibers ......................................................................... 20

1.10 Summary and conclusions ........................................................................................................................... 22

1.11 Acknowledgements ...................................................................................................................................... 23

References ............................................................................................................................................................. 24

Chapter 2: Release Profiles of Tricalcium Phosphate Nanoparticles from Poly (L-lactic acid) Electrospun Scaffolds with Single Component, Core-Sheath, or Porous Fiber Morphologies ................................................................................................................................. 36

2.1 Introduction ........................................................................................................................................................ 37

2.2 Materials and Methods .................................................................................................................................. 40

2.3 Results and Discussion .................................................................................................................................. 44

2.4 Conclusions ....................................................................................................................................................... 53

2.5 Acknowledgement ........................................................................................................................................... 54
Chapter 3: Novel, Silver Ion Releasing Nanofibrous Scaffolds Exhibit In Vitro and In Vivo Antibacterial Efficacy without the Use of Silver Nanoparticles

3.1 Introduction
3.2 Materials and methods
3.3 Biocompatibility assessment of scaffolds
3.4 In vivo evaluation of antimicrobial scaffolds
3.5 Statistical analyses
3.6 Results and discussion
3.7 Biocompatibility assessment of scaffolds
3.8 In vivo evaluation of antimicrobial scaffolds
3.9 Conclusions
3.10 Acknowledgements

Chapter 4: Skin Tissue Engineering for the Infected Wound Site: Biodegradable PLA Nanofibers and a Novel Approach for Silver Ion Release Evaluated in a Co-Culture System of Keratinocytes and Staphylococcus aureus

4.1 Introduction
4.2 Materials and methods
4.3 Results
4.4 Discussion
4.5 Acknowledgements
Chapter 5: Ibuprofen Loaded PLA Scaffolds for Wound Healing Application  

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Introduction</td>
<td>115</td>
</tr>
<tr>
<td>5.2 Materials and methods</td>
<td>117</td>
</tr>
<tr>
<td>5.3 Scaffold characterization</td>
<td>118</td>
</tr>
<tr>
<td>5.4 <em>In vitro</em> analyses with human epidermal keratinocytes</td>
<td>120</td>
</tr>
<tr>
<td>5.5 <em>In vivo</em> evaluation of 20% ibuprofen loaded bandages</td>
<td>121</td>
</tr>
<tr>
<td>5.6 Results</td>
<td>123</td>
</tr>
<tr>
<td>5.7 Discussion</td>
<td>132</td>
</tr>
<tr>
<td>5.8 Conclusions</td>
<td>135</td>
</tr>
<tr>
<td>References</td>
<td>137</td>
</tr>
</tbody>
</table>

Chapter 6: Summary and Conclusions  

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 Conclusions</td>
<td>139</td>
</tr>
<tr>
<td>6.2 Recommendations for future research</td>
<td>142</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 2.1  Solution properties and electrospinning parameters  ......................  41
Table 2.2  Surface area of different fiber structures  .................................  47
Table 3.1  Treatment protocol at each wound site (8 wounds/pig on each of 5 pigs, n=5) ..........................................................  68
Table 3.2  Staphylococcus pseudintermedius concentrations in control and treated wounds .................................................................  82
Table 3.3  Inflammation scoring of control and treated wounds .................  83
Table 4.1  Design of experiment for antimicrobial and cytotoxicity evaluation of scaffolds either separately or in a co-culture system  ..................  96
Table 5.1  Fiber diameter of 10, 20 and 30wt% ibuprofen loaded PLA bandages ...  124
Table 5.2  Regenerated skin thickness and number of blood vessels formed .......  131
LIST OF FIGURES

Figure 1.1 Examples of different release profiles ........................................... 6
Figure 1.2 Different parameters that can be applied to control the release of drugs from nanofibers ................................................................. 9
Figure 2.1 SEM micrographs of single component PLA fibers doped with TCP .... 45
Figure 2.2 SEM (a,b) and TEM (c) micrographs of core sheath PLA fibers ......... 45
Figure 2.3 SEM micrographs of porous PLA fibers at 500X magnification (a), 2000X magnification (b) and 10000X magnification (c) ............... 46
Figure 2.4 SEM micrographs of porous PLA fibers loaded with TCP nanoparticles at 500X magnification (a), 2000X magnification (b) and 10000X magnification (c) ........................................ 47
Figure 2.5 Release profile of different fiber structures ..................................... 48
Figure 2.6 In vitro weight loss as a function of time for different fiber structures ... 51
Figure 2.7 Viability images of human adipose derived stem cells (hASC) seeded on TCP-loaded electrospun scaffolds comprised of single component (a,d), porous (b,e), or core sheath (c,f) fibers ......................... 52
Figure 2.8 Cell differentiation on different scaffolds ................................. 53
Figure 3.1 Open and closed linear full thickness skin incisions on the back of a pig after placement of a scaffold under dermis layer ......................... 68
Figure 3.2 SEM micrograph of PLA nanofibers ........................................ 70
Figure 3.3 XPS spectra for PLA nanofibers coated with Silvadur ET (carbon and oxygen are components of PLA, nitrogen and silver are components of Silvadur ET) .................................................. 71
Figure 3.4 Release profile of silver from PLA scaffolds coated with Silvadur ET containing different concentrations of silver varying from 15.75 µg/ml (bottom) to 125 µg/ml (top) .................................................. 72
Figure 3.5  Antimicrobial properties of scaffolds treated with Silvadur ET containing 32.25µg/ml silver on E. Coli (a), Staphylococcus aureus (b), and silver resistant E. Coli (c) bacteria as evaluated via AATCC 147 test ................................................................. 73

Figure 3.6  Qualitative antimicrobial test (AATCC 147) for scaffolds coated with different concentrations of Silvadur ET ................................................................. 73

Figure 3.7  Quantitative antimicrobial assessment (AATCC 100) of scaffolds coated with Silvadur ET containing 250 µg/ml silver in Silvadur ET coating solution ................................................................. 74

Figure 3.8  Viability of human dermal fibroblasts on control (no silver, pure PLA alone) and antimicrobial (31.25 ug/mL silver in Silvadur ET coating of PLA fibers) scaffolds on days 1, 4 and 7 ........................................ 75

Figure 3.9  Viability of human epidermal keratinocytes on control (no silver, pure PLA alone) and antimicrobial (31.25 ug/mL silver in Silvadur ET coating on PLA fibers) scaffolds on days 1, 4 and 7 ........................................ 76

Figure 3.10  AlamarBlue reduction for human dermal fibroblasts seeded on different scaffolds indicated that 31.25 ug/ml Silvadur ET concentration did not affect cell proliferation relative to 0 ug/ml control .................................................. 77

Figure 3.11  AlamarBlue reduction for human epidermal keratinocytes seeded on scaffolds containing different silver concentrations indicated that 31.25 ug/ml Silvadur ET concentration did not affect cell proliferation relative to 0 ug/ml control .................................................. 78

Figure 3.12  SEM micrographs of human dermal fibroblasts seeded on pure PLA scaffolds (a, b, c), PLA antimicrobial scaffolds coated with Silvadur ET containing 31.25 µg/ml (d, e, f), 62.5 µg/ml (g,h, i), 125 µg/ml (j, k, l) and 250 µg/ml (m, n, p) silver at different magnifications of 100X, 500X, and 2000X (magnification increasing from left to right) ............ 79

Figure 3.13  SEM micrographs of human epidermal keratinocytes on antimicrobial scaffolds coated with Silvadur ET containing 15.75 µg/ml (a, c), 32.25 µg/ml (b, d), and 62.5 µg/ml (c, e) at different magnifications of 500X, and 2000X (magnifications increasing from top to bottom) ............ 80
Figure 3.14  Closed (a,c) and open (b, d) wounds inoculated with Staphylococcus bacteria with (a,b) and without (c,d) antimicrobial scaffolds at day 3 ...

Figure 4.1  SEM micrograph of the surface of highly porous silver microparticles ..

Figure 4.2  Co-culture system to evaluate human skin and/or other mammalian cells in combination with bacteria on three-dimensional nanofibrous scaffolds .................................................................

Figure 4.3  PLA nanofibers containing silver nanoparticles (a,b,c) or highly porous silver microparticles (d,e,f) .................................................................

Figure 4.4  Release profiles of silver ions from PLA scaffolds loaded with silver nano- or microparticles .................................................................

Figure 4.5  Human epidermal keratinocyte proliferation on scaffolds without bacteria in the culture medium .................................................................

Figure 4.6  Viability of human epidermal keratinocytes on scaffolds (Pure PLA: a,b,c, PLA containing silver nanoparticles: d, e, f, and PLA containing silver microparticles: g, h, i) at day 1 (a,b,c) and day 3 without (d,e,f) and with (g,h,i) presence of bacteria .................................................................

Figure 4.7  SEM micrographs of human epidermal keratinocytes seeded on scaffolds (a,d: pure PLA, be,e: silver nanoparticle loaded, c,f: silver microparticle loaded with (d,e,f) and without (a,b,c) presence of S. aureus at day 3 .................................................................

Figure 4.8  Human epidermal keratinocyte DNA quantitation on scaffolds both in the presence and absence of S. aureus bacteria .................................................................

Figure 4.9  S. aureus bacterial growth on acellular or human epidermal keratinocyte-seeded scaffolds .................................................................

Figure 5.1  Chemical formula of (S)-(+) Ibuprofen .................................................................

Figure 5.2  SEM images at 5000X magnification of ibuprofen loaded PLA bandages in weight percent of (a) 10%, (b) 20%, and (c) 30% ibuprofen .................................................................
Figure 5.3  H NMR spectra from top to bottom of pure PLA, 10, 20, 30 weight percent ibuprofen, and pure ibuprofen labeled with unique PLA (P) and Ibuprofen peaks (I) ................................. 124

Figure 5.4  Controlled release of ibuprofen loaded PLA bandages at both room temperature and 37°C (body temperature) ................................................. 125

Figure 5.5  AlamarBlue assay results for control PLA and 10, 20 and 30% ibuprofen loaded PLA bandages over a 14 day experimental duration – bars with different letters represent significant difference (p < 0.05) … 126

Figure 5.6  Live/dead images of HEK on PLA control and 10, 20 and 30% ibuprofen loaded PLA scaffolds ......................................................... 127

Figure 5.7  Representative images of healing of incision wounds on the backs of nude mice over 14-day period with/without scaffolds (acellular or seeded with human epidermal keratinocytes) .............................. 128

Figure 5.8  Contraction of wounds with/without scaffolds (acellular or cell seeded) - bars with different letters represent a significant difference ............ 129

Figure 5.9  Hematoxylin and eosin staining of cell seeded scaffold 14 days after placement on wound site – cells are present on both the surface of scaffolds as well as migrated through the pores ................................. 130

Figure 5.10  Hematoxylin and eosin staining of harvested skin samples from control wound sites without scaffolds (a,d), with acellular scaffold (b,e) and with HEK seeded scaffold (c, f) – the bottom pictures are the magnified areas in yellow rectangular in top pictures .................................................. 130

Figure 5.11  Representative images of immunofluorescent stained regenerated skin on wounds with no scaffold (a), with acellular scaffold (b), or with cell seeded scaffold (c) – keratin 10 is stained epidermis in green and Ki 67 stained proliferating cells in pink (arrows) ................................. 132
Chapter 1

Nanofibrous drug carriers for wound healing and tissue engineering applications: Modern strategies for controlled release

Controlled release of drugs such that drug concentration is maintained at an optimal therapeutic level is an active area of investigation with significant clinical impact. Nanofibers, with morphological characteristics resembling the native extracellular matrix (ECM), hold great potential as controlled release systems for wound healing and tissue engineering applications. In order to control drug release from nanofibrous scaffolds, many strategies have been developed and investigated. This paper provides a comprehensive review of the different approaches for controlled drug release from nanofibrous scaffolds. We propose that by understanding mechanisms of release and the influence of key parameters associated with specific drugs, fibers, and release media, functional nanofibers with controlled release capabilities can be developed that will address a multitude of critical clinical applications for wound healing and tissue engineering.
1.1 Introduction
Conventional drug delivery systems often increase the concentration of a drug in the site of action more rapidly than desired (1). This can cause biotoxicity or a rapid decrease in drug concentration, potentially limiting the therapeutic effect and necessitating frequent dosing (1). Controlled release techniques can improve the pharmacokinetic profile of a released drug by maintaining its level at an optimal therapeutic value during the healing period (1). Recently, “smart” drug carriers have gained widespread interest for wound healing and tissue engineering applications (2). A new generation of medicated wound dressings capable of releasing chemicals with therapeutic value, such as antimicrobials, growth factors, and other compounds to accelerate the healing process is being developed (3). In tissue engineering, novel biomimetic scaffolds with extended functionality and bioactivity that precisely deliver signaling molecules are being implemented to improve tissue repair and regeneration (4).

Advances in material design and engineering have led to development of novel functional materials for drug delivery systems in the form of solid foams, nanofibrous matrices, microspheres, and hydrogels (5). Hydrogels are one of the most popular scaffolds for delivery of cells and growth factors. They are highly absorbent polymers that have a degree of flexibility comparable to native tissue due to their high water content (6). However, hydrogels typically have poor mechanical properties and rapid degradation profiles that can limit their applications in tissue engineering (6, 7). Further, the large pore sizes and high water content of most hydrogels usually result in a relatively rapid drug release over time, an undesirable outcome for controlled drug delivery systems (6). Nanofibrous scaffolds are another well known class of drug carriers that have been extensively used in wound healing
and tissue engineering applications (8). Ultra fine nanofibers with their nanoscale diameters have morphological characteristics similar to the native extracellular matrix (ECM) surrounding cells in vivo (9). Such scaffolds promote protein adsorption, cell attachment and proliferation (10) and can be manipulated for specific mechanical properties (11, 12). The release of drugs entrapped within nanofibers can be modified to obtain a desired controlled release profile, unless the blend of drug and polymeric fiber is fully integrated at the molecular level (13).

Although there are a number of techniques available for synthesis of nanofibers including phase separation (14) and self-assembly (15), electrospinning is the most widely studied method (16). Electrospinning provides a simple, cheap, yet powerful solution for nanofiber synthesis. A typical electrospinning system usually consists of a metallic needle attached to a syringe filled with polymer solution, a grounded collector, and a high voltage power supply (17, 18).

Literature describing the use of electrospun nanofibers for drug delivery applications is vast and publications number in the hundreds. There are multiple reviews summarizing the results of these different studies with respect to a specific aim. Some of these reviews focus on categorization of polymers that have been used to develop nanofiber drug carriers (19, 20) and different drugs that have been encapsulated within nanofibers (21). The majority of reviews emphasize the use of nanofibers for specific applications such as wound healing (22-24) and tissue engineering (25-27). Although multiple systems for controlled drug release have been described in this literature base, a complete and thorough summary on all published techniques for controlling the release profile of drugs out of electrospun nanofibers
is not available. In this review, we attempt to provide such a summary with specific focus on novel work that has been done using nanofibers as controlled release systems for wound healing and tissue engineering applications. We classify what we consider to be the most critical parameters for achieving optimization of release from these systems (Figure 2) and conclude with a brief review of some novel, recent technologies that have been developed to achieve more complicated drug release profiles.

1.2 Controlled release nanofibers for tissue engineering applications

The fibrillar structure of collagen within the extracellular matrix is known to be important for cell attachment, migration, proliferation, and differentiation (28). Polymeric nanofibrous mats, by mimicking the morphological characteristics of collagen, may lead to generation of engineered tissue more closely resembling native tissues (29). Furthermore, nanofibrous scaffolds releasing exogenous signaling molecules upon local cellular demand, could lead to creation of novel multifunctional materials that are capable of controlling the developmental process in tissue and organ specific differentiation and morphogenesis (30-35). However, due to the sensitivity of cells to the concentration of released compounds, successful application of doped scaffolds for tissue engineering applications greatly depends upon accurate control of release kinetics.

1.3 Applications of controlled release nanofibers in wound healing

Wound healing is a complicated process comprised of hemostasis (for bleeding wounds) inflammation, proliferation or granulation, and maturation or remodeling (36). These phases can vary depending upon the wound type, size, depth, location and presence of infection (36). Modern wound dressings are primarily designed to address a specific wound type to enhance
the healing process. The dressings tend to keep the wound bed moist to stimulate cell proliferation and encourage re-epithelialization, resulting in an accelerated healing process.

Wound dressings can also reduce pain, provide autolytic debridement, and act as a bacterial barrier (37). Many incorporate drugs of interest to further assist with the healing process and reduce the frequency of wound dressing changes (22). Degradable doped dressings can also be washed from the wound surface once they have released their encapsulated drugs (3). Novel dressings such as these could be of great benefit for the treatment of chronic wounds for patients undergoing long wound care treatments. Desired release patterns of drugs in different wound types have been previously presented by Leung et al. (Figure 1.1(a)) (22).

Nanofibers with appropriate mechanical properties, porosity, and moisture pick-up, have been reported to be a desirable option for wound care as they can easily be doped with different hydrophobic and hydrophilic drugs of interest as needed for specific applications. Previous investigators have also modified nanofibers to obtain desired release patterns (38-40). However, controlled temporal and quantitative release of drugs to address the needs in each healing phase (hemostasis, inflammation, proliferation, and maturation) remains an unsolved challenge.
Figure 1.1: Examples of different release profiles [22, 58, 73, 90, 110]; (a) examples of release profiles for different applications: antibiotics for fresh wounds (---), drugs for surgical wounds (--), drugs for epithelialized wounds (- - -) (adapted from Leung et al. [22]). (b) common release profiles: burst release (---), controlled release (--), delayed release (- - -) (adapted from Jiang et al. [90]). (c) release of drugs from core-sheath nanofibers relative to core to sheath ratio (adapted from Jannesari et al. [58]). (d) effect of scaffold thickness on release behavior of dopants from electrospun nanofiber mats (Adapted from Abidian et al. [110]). (e) cumulative mass release of dexamethasone from: PLGA nanoscale fibers (---), PEDOT-coated PLGA nanoscale fibers without electrical stimulation (- - -), and PEDOT-coated PLGA nanoscale fibers with electrical stimulation of 1 V applied at specific times indicated by the arrows (---) (Adapted from Okuda et al. [73]).(f) release profile of tetra-layered drug loaded nanofiber meshes: release profiles of drug from first layer (---) and third layer (- - -).
1.4 Release mechanisms

To date, a variety of different nanofiber systems have been developed as drug carriers capable of releasing drugs over a prolonged period of time. The release of drugs from nanofibers follows different patterns depending on the individual system. There are predominantly three different release profiles for encapsulated drugs in nanofibers: 1) burst release, 2) controlled release, and 3) delayed release (Figure 1.1(b)). When the majority of drug is released from the nanofibers shortly after placement in release medium without achieving a stable release rate, the drugs have undergone a “burst release”. Although the burst release profile can be favorable for some limited purposes such as targeted delivery, pulsatile release, or some specific wound dressings, it is usually undesirable and many investigators have attempted to find methods to prevent burst release (41-45). The main disadvantages of the rapid initial release of drugs include toxicity caused by high drug concentration, rapid loss in drug affinity, and an increase in dosing frequency (46). The “controlled release” profile is usually the most favorable release pattern because it gradually releases the drugs at a controlled rate. When the controlled release pattern follows a linear model as a function of time it is known as a “zero-order controlled release” (47). The third most common release profile for nanofibrous drug carriers is the “delayed release” profile in which the drug is released at a later point after initial administration. This system can be used with drugs that need to be released at specific, delayed time points (48).

Depending on the final application of the doped nanofibers, one or even a combination of more than one of these release profiles may be desirable. In order to achieve a desired release pattern of drugs from nanofibers, it is necessary to clearly understand and
manipulate the release mechanisms that influence release rates. Typically, the release of an encapsulated compound from non-degradable polymeric fibers has been attributed to solid-state diffusion. Fick’s Law of diffusion provides the basis for the clarification of solute transport from polymeric matrices (49). As opposed to what was widely assumed before, it has been shown that the release rate from nanofibers does not always follow theoretical predictions based on solid-state diffusion (50). In many experiments a complete release of drugs does not occur after extended duration in aqueous medium (51-53) even though according to the principles of solid-state diffusion 100% release should have been achieved. Recently, investigators have proposed another mechanism, “desorption-limited”, to explain the release of compounds from nanofibers (54, 55). According to this mechanism, the entrapped compounds within nanofibers desorb from the surfaces of the fibers and through nanopores occurring throughout the fiber bulk that are in direct contact with the release medium (e.g. water). After this desorption, the desorbed compounds quickly diffuse into water with the release rate determined by the limited-desorption phase. Yet another mechanism that facilitates the release of drugs is degradation, which can follow either a bulk or surface degradation profile. Bio-degradation of polymeric fibers results in greater exposure of the drug molecules to the aqueous environment surrounding the nanofibers. Incorporation of bio-degradable polymers for delivery of compounds that dissolve at a slower rate compared to the polymeric matrix is a versatile method (56).

The relative effect of each of these mechanisms on release rate depends upon material choice and fiber morphology. Consequently, the rate of drug release can hypothetically be
precisely controlled to match an ideal pattern for a specific application by regulating the activity level of one or more of these mechanisms.

Strategies to control the release of drugs from nanofiberous scaffolds

- **Nanofiber Scaffold**
  - Polymer composition
  - Fiber Morphology
  - Thickness of scaffold

- **Drug**
  - Concentration
  - Solubility
  - Hydrophilicity

- **Release Medium**
  - Surface tension
  - Temperature
  - pH & Enzymes

**Figure 1.2:** Different parameters that can be applied to control the release of drugs from nanofibers

### 1.5 Drug properties

**Drug concentration**

There are multiple publications investigating the effect of drug concentration on the release profile of drugs from monolithic nanofibers (43, 57-59). It has been reported that when drug concentration is lower than the solubility limit of the polymer, the drug will exhibit a slower release rate. For high drug concentration where the concentration of the drug is above the solubility limit of the polymer, the release profile is that of a large burst
release followed by a shorter period of gradual, controlled release. Therefore, for long term release applications where a high drug concentration is desirable, there needs to be a balance between the amount of loaded drug and its release rate that appear to vary in parallel directions. We have doped poly (L-lactic acid) (PLLA) fibers with different concentrations of tricalcium phosphate (TCP) nanoparticles and analyzed the effect of release on osteogenic differentiation of human adipose derived stem cells (hASC) (60). We have shown that the greatest release occurs at the first time point of analysis (day 6) with increasing release occurring with higher concentration of doped nanoparticles at all time points, as expected. Osteogenic differentiation analyses of hASC indicated higher osteogenic differentiation for hASC grown on scaffolds doped with elevated TCP nanoparticle concentrations with osteogenic differentiation increasing in both atime and dose responsive manner (60).

**Drug solubility**

The solubility and compatibility of the drug in a drug/polymer/solvent system are the critical factors for preparation of an electrospun fiber formulation with a constant release profile. Natu et al. doped polycaprolactone (PCL)/ Lutrolnanofibers with timolol maleate and acetazolamide with water solubility of 2.74 mg/ml and 0.98 mg/ml, respectively (57). Their findings indicated that acetazolamide had higher solubility in fibers relative to timolol maleate. Timolol maleate also released faster than acetazolamide when doped in the same type of fibers at similar loading concentrations, indicating that drug solubility in the polymer influenced the partition of drug between polymer and elution medium (57). More investigation seems to be needed to completely understand the effects of drug solubility on the release profile of nanofibers.
**Drug hydrophilicity**

In order to encapsulate the majority of a drug inside polymeric fibers, and thus acquire a constant and stable drug release profile, a lipophilic polymer should be chosen as the fiber material for a lipophilic drug (21). Similarly, a hydrophilic polymer should be employed for a hydrophilic drug, and the solvents used should be suitable for both drug and polymer (21). This applies to drugs to be released from polymeric systems; however, hydrophilic drugs can also be encapsulated in microspheres and suspended in polymeric solutions (61) or used as the core of core-sheath fibers (43, 62).

### 1.6 Nanofiber scaffolds

**Polymer composition**

Polymer choice is critical for delivery of compounds for biomedical applications. Biocompatibility is a requirement for biomaterials and depending on the materials’ final application, biodegradability may also be desired. The use of non-degradable polymers in tissue engineering applications often requires an additional surgery to harvest the polymeric scaffold from the body once the drug is depleted from the scaffold. Thus, non-degradable polymers are more suitable for applications in which removal of the implant is not difficult (e.g. ocular implants) (63). Conversely, it is not necessary to remove degradable polymers, so they have been extensively studied for drug delivery applications (64). However, since degradable polymers break down to absorbable molecules, it is essential to ensure that the monomers are non-toxic. Degradation of hydrolytically degradable polymers generally depends on their chemical composition, crystallinity, molecular weight, and hydrophilicity (65). Chemical composition determines the type of degradable bonds present in
a polymer. Biodegradable polymers have hydrolysable bonds such as glycosides, esters, orthoesters, anhydrides, carbonates, amides, urethanes and ureas (65, 66). Generally, anhydride bonds degrade faster than ester bonds and amide bonds have an even lower rate of degradation.

Biodegradable polymers are either semi-crystalline or amorphous and initial hydrolysis occurs at the amorphous regions due to these regions’ higher accessibility to water molecules and enzymes. Therefore, it is typical to have higher degradation rates in less crystalline polymers. Zilberman investigated the effect of the degree of crystallinity on the release profile of dexamethasone from PLLA and poly(DL-lactic-co-glycolic acid) (PDLGA) films (67). He concluded that a high degree of crystallinity decreases the rate of drug release but better maintains desired mechanical properties. According to this study, the effect of crystallinity on release rate diminishes as weight loss increases. In high weight losses, when the film gets a porous structure, the crystallinity does not have a significant effect on drug release. Dias et al. studied the degradation of electrospun PLLA fibers with different crystallinities (ranging from 0 to 45%) (68). They showed that the degradation of PLLA in phosphate buffered saline (PBS) caused greater reduction in molecular weight for samples with lower crystallinity (68). Poly lactic-co-glycolic acid (PLGA), a copolymer of PLA and poly glycolic acid (PGA), is one of the most commonly used polymers in drug delivery applications and has been thoroughly reviewed by Makadia and Siegel (69). They indicated that the hydrolysis and biodegradation rate of PLGA to undergo hydrolysis and the biodegradation rate of the polymer are directly influenced by the degree of crystallinity of
PLGA. The higher the content of PGA in PLGA, the lower the degree of crystallinity and the quicker the degradation (69).

Polymers that contain many hydrophobic groups usually degrade slower than hydrophilic polymers. Subsequently, the release of encapsulated drugs from these polymers is slower. The most common synthetic polymers electrospun into nanofibers for biomedical applications include some of the hydrophobic biodegradable polyesters, such as PGA, polylactic acid (PLA) and PCL. In order to accelerate release of drugs from these biomaterials, polymers that are hydrophilic in nature such as poly vinyl alcohol (PVA) and poly ethylene oxide (PEO) are sometimes mixed with these hydrophobic polymers. Blending and copolymerization are two useful methods for mixing different polymers to obtain new material properties. Fathi-Azarbayjani et al. have shown that the release of haloperidol can be changed by controlling the hydrophilicity of the encapsulating nanofibers (70). They used blends of hydrophilic polymers, PVA and RM β-CD, and hydrophobic polymers, PLA and PLGA. Since hydrophilic polymers can dissolve in release media almost instantaneously, the addition of these hydrophilic polymers to PLA and PLGA allowed faster release of the encapsulated drug. They concluded that blending hydrophilic and hydrophobic polymers can be useful for treatment of acute disorders where a rapid release of drugs is needed, as opposed to chronic disorders that require gradual release of drugs. Blends of poly (D-lactic co-glycolic acid) (PDLLA) and PEO with different ratios (PEO:PDLLA 90:10, 70:30, 50:50) have also been studied for encapsulation of two bacteriocins (Plantaricin 423 and bacteriocin ST4SA) in electrospun nanofibers (71). That study also reported a greater release with higher amounts of the hydrophilic polymers. However, since the ratio of PEO to
PDLLA was high, the initial release was very fast for all samples and a burst release pattern was observed for all ratios of PEO to PDLA that were analyzed. Finally, yet another polymer related parameter that can directly influence the release rate of encapsulated drugs is molecular weight. Several studies have shown that compounds encapsulated in polymers with greater molecular weights exhibit a slower release rate (53, 54, 72).

1.7 Fiber morphology

Fiber diameter and porosity of scaffold

Fiber diameter greatly influences the surface area of the fabricated web (the larger the fiber diameter, the less the surface area). Release rate depends upon the surface area in which the fibrous scaffold interacts with the release medium. Finer fibers having a larger surface area to volume ratio will therefore release their dopants faster (73, 74).

Surface morphology of fibers

Surface morphology of fibers can influence wettability and degradation properties and thus the release rate of encapsulated drugs from fibrous scaffolds. Most surface modifications for nanofibers previously reported in the literature have focused on development of pores of different sizes on the fibers. There are a few methods for producing electrospun fibers with pores on the fibersurface. The most versatile methods are selective removal of a component from bicomponent fibers (75), phase separation during electrospinning (76), addition of a second volatile solvent (77), and use of a single highly volatile solvent (78, 79). Porous fibers increase both the surface area and the porosity of the scaffold providing not only a good method to regulate drug release but also potentially enhance cell attachment and migration sites. Previous investigators have utilized porous
fibers doped with various drugs for wound healing and tissue engineering applications (79-82). Porous PLA fibers prepared using a phase separation technique have been doped with ketoprofen (1%, 3%, 5%, 8%), a non-steroidal anti-inflammatory drug (81). It was reported that the porosity of the fibers decreased as ketoprofen concentration in fibers increased such that fibers containing greater than 1% drug content appeared to have a smooth structure. Interestingly, the percent drug release was reported to be considerably higher for fibers doped with 1% ketoprofen relative to all other concentrations evaluated. This was related to the surface porosity of the fibers which determined the specific surface area of the fibrous scaffold (81). Similarly, Moroniet al. introduced nanoporosity to the surface of solid poly(ethylene oxide terephthalate)–poly(butylene terephthalate) fibers containing methylene blue dye and observed a significant increase in the dye release rate (74). In our lab, we have developed porous PLA fibers using a highly volatile solvent and doped them with tricalcium phosphate (TCP) nanoparticles for bone tissue engineering applications (79). Traditional single component PLA fibers, having a solid surface morphology, also were doped with TCP nanoparticles as the control. The release of TCP nanoparticles from fibers with these different surface morphologies was quantified over 36 days. Although the results revealed a burst release profile for both fiber types, the initial release rates were significantly different, with a higher rate occurring with the porous fibers (79). This outcome was expected due to the higher diffusional and degradation properties of porous fibers (79).
Cross-sectional fiber characteristics

Localization of drugs within a specific length in the cross section of fibers plays an important role in controlling the release rate of drugs from fibrous scaffolds. When dopants are closer to the fiber surface, there is a greater chance for quick release. Electrospun core-sheath fibers provide the ability to load desired drugs in either the outer layer (sheath) of the fiber to be released faster or in the inner part (core) for a slower release over time (83, 84). The technology of co-axial electrospinning for creating core-sheath nanofibers, first reported by Sun et al. (85), is similar to conventional electrospinning with the exception that the single needle is replaced by a pair of co-axially aligned capillaries that are both charged and deliver two polymer solutions simultaneously.

Most studies using core-sheath fibers have encapsulated drugs within the core and have reported a sustained release profile which, in many cases, occurred after a short term burst release (84, 86-89). In our lab we have encapsulated TCP nanoparticles in the core of core-sheath fibers composed of PLLA. Our results showed a slow steady release of calcium ions from the core sheath fibers as opposed to the initial burst release from the single component PLLA fibers (79). The release mechanism for core-sheath fibers is predominantly a function of the properties of the sheath polymer as the drug will be released as the sheath polymer degrades. Therefore, incorporating polymers with higher degradation rates for the sheath increases the release rate of the dopant. A review paper by Yarin indicated that imperfections such as cracks and pores in the sheath are the reason that drugs are released when the sheath polymer is not biodegradable (83). In these cases, addition of
materials that can rapidly dissolve in water and leave pores in the sheath (e.g. polyethylene glycol (PEG)) is helpful for increasing rate of release when this is desired (90, 91).

The emergence of coaxial electrospinning also allows for nanofibers to encapsulate hydrophilic drugs and bioactive agents that may be challenging to dope in monolithic fibers such as peptides, growth factors (92, 93), proteins (90, 94) and even DNA (95, 96) and siRNA (97, 98). Incorporation of an aqueous solution of bioactive compounds in the core of such fibers limits physical contact between the bioactive agent and the sheath solution, which may contain harsh solvents, to only the boundary between the core and sheath. Further, the core is protected from the high voltage necessary for electrospinning since the electric charge will rapidly move to the outer layer of the sheath as the jet is formed (99). The spinnability of the core solution can also be improved with the addition of a small amount of a water soluble polymer such as PEO, PVA and PEG.

Another advantage of electrospun core-sheath fibers is the ability to program their release kinetics by control of the sheath thickness. Many studies have shown that, as expected, a thicker sheath delays the release of core components (84, 90, 100). The thickness of the sheath can be adjusted by altering the concentration of sheath solution or the solution feed rates. He and co-workers entrapped tetracycline hydrochloride (TCH) in the core of fibers comprised of different concentrations of PLLA in chloroform and acetone (100). A sustained release for all different sheath concentrations was reported. The more concentrated PLLA solution (10%) resulted in creation of thicker fibers which further reduced release percentage over 30 days (100). The effect of feed rate on sheath thickness has also been studied (90). Jiang et al. incorporated two model proteins, bovine serum albumin (BSA) and
lysozyme, in combination with PGA in the core of PCL fibers. By changing the feed rate of the core solution the thickness of both the core and sheath changed. The higher feed rate speed resulted in a smaller ratio of sheath to core and subsequently a faster release of protein (Figure 1.1(c)) (90).

1.8 Thickness of scaffolds

The thickness of electrospun nanofiber webs can be easily controlled by the duration of electrospinning. It has been reported that thicker nanofiber webs release their encapsulated drugs more slowly as compared to the same webs of lesser thickness (Figure 1(d)) (58). This phenomenon has been related to the more compact structure of thicker webs that can physically diminish the release of drugs (58). More study is still needed to understand the effect of scaffold thickness on drug release.

Releasing medium

Tissue engineered scaffolds are ultimately being used at body temperature and pH. Therefore in vitro experiments should be designed to mimic as closely as possible the in vivo environment. The release behavior of a polymeric nanofiber is influenced by temperature, pH, and other release medium properties. A brief description of the different parameters of release medium that can influence drug release is explained below.

Surface tension of releasing medium

Maretschek et al. (2008) electrospun PLLA with an aqueous protein solution of Cytochrome Cand found that the release of protein in phosphate buffered saline (PBS) was very slow due to the hydrophobic properties of the PLLA fibers (43). An emulsifier, Tween 20, was added to the PBS to decrease the surface tension of the releasing medium. With this
addition, the surface tension of PBS was decreased, increasing the wettability and the release of protein (43). Leelarasamee et al. also reported that addition of surfactants such as polysorbate 80, cetylpyridinium chloride, and aerosol OT in the releasing medium (PBS, pH: 7.4) largely increased the release rate of hydrocortisone from polylactic acid microcapsules(101).

Temperature of releasing medium

The release of drugs from nanofibers is usually measured at either room temperature (21 °C) or body temperature (37 °C). Previous studies have shown that the rate of release is significantly higher at body temperature as compared to room temperature(81, 102). This has been attributed to the diffusion dependency of the release as temperature increases the diffusion rate(103). Although the role of temperature of the release medium has not been thoroughly studied in the context of nanofibrous drug carriers, there are a few studies investigating the effect of release medium temperature on the release of dopants from polymeric microparticles (104-106). Findings from those studies can likely be correlated to the release of drugs from nanofibers since the same release mechanisms apply to both of these drug carriers. Shameem et al. studied the release of leuprorelin from PLGA-based microparticles into a phosphate buffer (pH 7.0) at temperatures ranging from 37 to 60 °C(105). According to their results, the drug release rate significantly increased with increasing temperature. This was due to faster degradation of PLGA at higher temperatures (104). Faisant et al. investigated the release of 5-FU from PLGA-based microparticles at 37, 45, 53, 60, and 65 °C in phosphate buffer (pH 7.4) and also concluded that the release rate significantly increased with an increase in the temperature from 37 to 65 °C. They
hypothesized this occurred as a result of the increase in the mobility of the polymer chains and drug molecules at higher temperatures, leading to elevated diffusion rates (106).

**pH and enzymes**

Meng et al. have shown that the release rate of drugs encapsulated in polymeric matrices can change depending upon the pH of the releasing medium. However, they noted that nanofibrous scaffolds are more stable than solvent-cast films in a medium with different pH values (107). Wang et al. doped four different anionic model drugs in poly(2,6-dimethyl-1,4-phenylene oxide) (BPPO) and determined the release rate of the drugs in both deionized water and phosphate buffered solutions (PBSs) with pH values of 5.0, 7.4, and 9.0. Their results indicated that the release of drugs is much faster in salty solutions with high ion concentration compared to deionized water. They explained that electrostatic interaction plays an important role in the release of anionic drugs in the anion exchange membranes and that hydrogen bonding between the drug and fiber is pH dependent (108). Similarly, Huang et al. have shown that the solubility of cellulose acetate phthalate (CAP) fibers, and so the release of anti-viral drugs loaded into them, is influenced by pH of the surrounding medium (109). They reported that fibers were stable in *simulated vaginal fluid* (SVF) (pH 4.2) as opposed to PBS (7.4) in which the fibers immediately dissolved and released their entrapped compounds within 2 minutes (109).

### 1.9 New strategies applied for controlled release nanofibers

In addition to all of the parameters described above that can be adjusted to control the delivery of compounds through electrospun nanofibers, other, newer strategies are being developed to promote specific drug release patterns for nanofibrous drug carriers. Abidianeet.
al. have proposed a method to precisely release drugs from electrospun nanofibers in a desired release profile using electrical stimulation (110). They have electrospun dexamethasone loaded PLGA nanofibers and then coated then with a conducting polymer(poly(3,4 ethylenedioxythiophene (PEDOT)) by electrochemical deposition. They reported a significantly lower rate of dexamethasone release for nanofibers coated with PEDOT compared to uncoated controls (Figure 1(e)). However by applying electrical stimulation to PEDOT nanotubes at specific time points, the release of dexamethasone from the coated nanofibers was abruptly increased (Figure 1(e)) (110). Electrical stimulation, induced by a positive voltage of 1 V, resulted in the contraction of nanofibers which created mechanical pressure within the nanotubes that then exuded the dexamethasone through pores or cracks formed by actuation on the surface of the nanotubes (110).

Another recent technique that has been utilized to control the release rate of drugs is sequential electrospinning to develop layered nanofibers (73, 111, 112). In this method, layers of drug-loaded nanofibers are covered with an additional nanofiber layer of either the same or a different polymer. The extra layer acts as a barrier and delays release of the encapsulated drugs. This facile method is also useful to more easily encapsulate hydrophilic drugs. Jian et al. loaded zoledronic acid in PEO nanofibers and sandwiched those between two layers of PLA nanofibers (112). The release of zoledronic acid from the three-layered scaffold followed a zero-order pattern. The release rate of zoledronic acid was successfully controlled by changing the thickness of the PLA barriers (thicker PLA layers slowed drug release). The same results were reported by Kim et al. when antimicrobial peptide HPA3NT3 was encapsulated in PEO with PCL nanofibers sandwiching the PEO nanofibers.
Okuda et al. investigated the feasibility of this technique for dual delivery of drugs for applications where sequential release of drugs is needed such as chemotherapy with release of multiple chemotherapeutic drugs released at different time points (73). They used two dye compounds as model drugs and doped them in the first and third layers of a tetra-layered nanofiber construct developed by sequential electrospinning. Their results showed a burst release for the dye loaded in the first layer whereas the dye in the third layer, which was protected by the two other nanofiber barrier layers, exhibited a delayed release profile (Figure 1(f))(73). The time period between releases of the two drugs was controlled by varying the thickness of the layers.

1.10 Summary and conclusions

Extensive progress has been made in the last decade to employ nanofibers as drug carriers for wound healing and tissue engineering applications. Successful use of nanofibrous drug carriers depends upon understanding of the proper release profile required for the final application; and, subsequently selecting the best polymer drug system along with a suitable strategy. In this paper, we have attempted to shed light on the many different, available techniques for controlling the release of drugs from nanofibers to enable researchers with strategic decisions for targeting a specific clinical need. As described in this review, nanofibrous drug carriers allow localized, controlled release of drugs through controlling specific parameters related to the scaffold itself, the encapsulated drugs, and the releasing environment.

However, despite the many efforts toward creation of smart nanofibrous drug carriers and new understanding of the important parameters involved, there is still an enormous need
for more studies to better control the release process. When targeting the human body, there must be the least possible error in the expected function of biomaterials, otherwise catastrophic consequences may occur. To date, as indicated in this review, the vast majority of investigations on drug releasing nanofibers have been performed *in vitro* with only a few successful evaluations *in vivo*, while the advances made have been significant, much work remains to be done to completely harness the promise of nanofibrous controlled delivery systems.

1.11 Acknowledgements

This work is supported by NIH/NIBIB 1R03EB008790, NSF/CBET 1133427, Nonwovens Cooperative Research Center (Project 10-128, EGL), and Chancellor’s Innovation Fund (EGL). The authors would like to thank and all other members of the Cell Mechanics Laboratory.
REFERENCES


71. Heunis, T., Bshena, O., Klumperman, B., and Dicks, L. Release of Bacteriocins from Nanofibers Prepared with Combinations of Poly(D,L-lactide) (PDLLA) and Poly(Ethylene Oxide) (PEO). International Journal of Molecular Sciences 12, 2158, 2011.


86. Sakuldao, S., Yoovidhya, T., and Wongsasulak, S. Coaxial electrospinning and sustained release properties of gelatin-cellulose acetate core-shell ultrafine fibres. ScienceAsia 37, 335, 2011.


Chapter 2

Release Profiles of Tricalcium Phosphate Nanoparticles from Poly (L-lactic acid)Electrospun Scaffolds with Single Component, Core-Sheath, or Porous Fiber Morphologies

Published in Macromolecular Bioscience, 12 (7), 893-900, 2012

Nanofibers have recently gained great interest for their potential as controlled release systems of benefit for wound healing, regenerative medicine and tissue engineering applications. Controlling the release rate of desired dugs or compounds during therapy is an active area of investigation. An appropriate way to regulate the rate of release from nanofiber scaffolds is to change the nanofiber's structural properties. In this study, functional poly-L-lactic acid (PLA) scaffolds created with single component, core-sheath, or porous fibers were created. The scaffolds were doped with tricalcium phosphate (TCP) nanoparticles for investigation of the doped scaffolds'release profiles for potential future use in bone tissue engineering applications. Pharmacokinetic analyses were performed for the three different nanofibrous structures after doping them with TCP. Results indicated that single component and porous fibers exhibited an initial burst release profile while core-sheath fibers exhibited a steady release rate. All nanofibrous scaffolds were then seeded with human adipose derived stem cells (hASC). Human ASC remained viable and continued proliferation on all nanofibrous morphologies for 21 days. Osteogenic differentiation of hASCs varied on the three fiber morphologies with the greatest cell mediated calcium accretion occurring on porous PLA nanofibers and the least on core-sheath fibers.
2.1 Introduction

Bone tissue engineering often incorporates the use of an artificial extracellular matrix, or scaffold, seeded with osteoblasts or cells that can become osteoblasts as a template for bone regeneration. Ideal scaffolds are biocompatible, biodegradable, and promote cellular interactions and tissue development, and possess proper mechanical and structural properties, including adequate porosity [1-4]. Several processing technologies, such as particle leaching [5], phase separation [6] and self-assembly [7] have been developed to fabricate porous scaffolds for bone tissue engineering applications. Effective mimicry of the structure of the natural extracellular matrix (ECM) has yet to be fully realized. Three dimensional (3D) structures produced by electrospinning contain submicron scale fibers with interconnected pores between fibers, resembling the topographic features of the natural ECM. The large surface to volume ratio of electrospun fibers also improves drug loading, mass transfer, cell attachment, proliferation and differentiation [8, 9]. Electrospinning is considered a versatile method to produce continuous fibers from the nanometer to submicron diameter using a broad range of polymers [10, 11]. In a typical process, a high voltage is applied to the solution, which is extruded from a spinneret to form a droplet. At a critical voltage, electric Maxwell stresses acting from the counter electrode overcome the surface tension of the polymer solution and cause a fine jet to erupt from the pendent droplet and accelerate toward a charged collector [12]. As the jet travels through air, the solvent evaporates and the chaotic nature of the electrospinning jet motion, or bending instability, causes the jet to be stretched many times beyond its original length to produce continuous ultra thin polymeric fibers in the form of nonwoven mats [13-15].
The convenience of incorporating various materials into the electrospinning process makes it attractive for tissue engineering applications. Loading a mineral component such as demineralized bone powder, hydroxyapatite, calcium carbonate and/or $\beta$-tricalcium phosphate (TCP) into electrospun scaffolds in order to permit and promote bone ECM production and ultimate ossification is an active area of investigation [16-18]. Previous studies in our lab have shown that the addition of TCP into single component electrospun scaffolds induces and accelerates osteogenic differentiation of human adipose derived stem cells (hASC) and encourages rapid ossification [19]. We have also found that an 8mM concentration of calcium ions in the culture medium greatly enhances hASC osteogenic differentiation and calcium accretion for hASC in monolayer culture seeded on tissue culture plastic [20]. However, PLA/TCP scaffolds created via a conventional electrospinning process exhibit an initial burst release profile of TCP nanoparticles from the “single component” fibers (i.e., "single component" polymeric fibers loaded with TCP as opposed to fibers having core-sheath or porous fiber morphology). With such a release profile, delivery of a constant concentration of calcium during osteogenic differentiation and bone formation by cells seeded on the scaffolds is not possible. Previous studies have described the advantage of sustained release at small amounts over a burst release; in particular the lower risk of sustained release leading to unanticipated side-effects [21, 22]. To date there has been no attempt to control the release rate of TCP from electrospun scaffolds. In this study we controlled the release rate of TCP nanoparticles from scaffolds using custom electrospinning systems that allowed us to vary both individual fiber morphologies and overall three-dimensional fibrous scaffolds.
A well known fiber structure for controlled delivery systems is the core-sheath fiber [23]. Ultrafine core-sheath electrospun fibers made of two miscible or immiscible polymeric solutions can be manufactured by coaxial electospinning [24]. The technology of co-axial electrospinning is similar to that used for spinning single component nanofibers, except a pair of capillaries that are co-axially aligned deliver two polymer solutions and that both charged simultaneously [25, 26]. This process leads to encapsulation of the core material which can be potentially advantageous for the storage and delivery of drugs.

Another method to control the release rate of drugs is via modification of the surface area of the nanofibrous mats. A greater surface area allows for higher release. An obvious advantage would be achieved by the creation and use of porous fibers, with their much greater surface area and sites for cell growth, proliferation, and differentiation. Different methods have been investigated for creation of porous fibers via electrospinning. These include phase separation, selective removal of a component from a blend of materials [27], addition of a second volatile solvent [28], and use of a single highly volatile solvent [29]. The latter method was implemented in this study to create porous fibrous structures that were manufactured in a one-step process.
2.2 Materials and methods

Scaffold Fabrication

In this study three different fiber morphologies (single component, core sheath, and porous) were developed and loaded with TCP nanoparticles. PLA, a biodegradable, biocompatible polymer with a molecular weight of 70,000 g/mol, was dissolved in chloroform and dimethyl formamide (DMF) (both obtained from Sigma, St Louis, MO) at a ratio of 3:1 to create a 12 wt% solution. Extra pure TCP (Ca$_3$(PO$_4$)$_2$) (Sigma, St Louis, MO), in the form of spherical crystals with an average particle size of 40 nm, was added to the PLA solution at a weight concentration of 10 wt%. Solutions were then stirred for 24 h and sonicated prior to electrospinning to disperse the TCP particles. This solution was then used to electrospin single component fibers.

To create core-sheath fibers, the same solution was pumped into an inner needle to form the core composite polymer. A solution of 12 wt% PLA in chloroform and DMF was utilized in the outer needle, forming the sheath polymer. Porous fibers were created using the identical PLA was used in creation of the single component and core-sheath fibers that was then dissolved in dichloromethane (DCM) (Sigma, St Louis, MO) to form a 12wt% solution. 10wt% TCP nanoparticles were added to the solution using the same approach described above for single component nanofibers loaded with TCP. Table 2.1 provides properties of the different polymeric solutions and the electrospinning parameters used to create each of the three distinct fiber morphologies.
Table 2.1: Solution properties and electrospinning parameters

Electrospun scaffolds were kept under a fume hood overnight to fully evaporate the solvent. The scaffolds were then peeled off the fiber collector (aluminum foil) and cut into roughly 10×10 mm² squares. The thickness of each scaffold was measured with a Mitutoyo absolute micrometer (Aurora, IL) and the average thickness from at least 30 measurements was calculated. The weight of each sample was measured and maintained within 30% of the mean average of all samples, 3 mg/cm².

Microscopic analysis
Field emission scanning electron microscopy (FESEM JEOL 6400 F) and transmission electron microscopy (TEM Hitachi HF2000) were used to characterize the surface morphology and microstructure of the ultra fine electrospun fibers at 15 kV and 200 kV accelerating voltage, respectively. Using SEM images, the average fiber diameter of each scaffold was calculated from at least 50 measurements per scaffold.

Surface area analysis
The surface area of different fiber morphologies was quantified using an Autosorb-1C apparatus equipped with the ASWin software package (Quantachrome Instruments, FL). Nitrogen adsorption/desorption isotherms were performed using 100mg of each sample and a 40-point adsorption/desorption scheme. Before analysis, the sample was allowed to outgas at the outgas port
of the Autosorb 1 for more than 4 h. Surface areas were calculated by applying Brunauer_Emmett_Teller (BET) theory over the linear region of the isotherm.

**In vitro calcium release**

The prepared scaffolds were sterilized in 70% ethanol (pH~7) for 10 minutes in room temperature. The short period of being exposed to ethanol in room temperature can not change the properties of scaffolds significantly. Sterilized scaffolds were soaked in 12 well plates filled with complete growth medium (CGM), consisting of α-MEM (88%), FBS (10%), L-glutamine (1%) and Pen/Strep (1%) and incubated under physiological conditions (37 °C, 5% CO₂). At time points of 1, 2, 4, 8, 12, 16, 20, 24, 28, 32 and 36 days, three scaffolds of each type were then removed from CGM and washed with PBS to ensure no released calcium remained attached to the scaffolds. To determine the amount of calcium remaining in the scaffolds, each scaffold was digested in 0.5 mL HCL overnight then removed and the supernatant used for calcium quantification via the Calcium Liquicolor Assay (Stanbio, Boerne, TX).

The amount of TCP initially doped within the scaffolds was determined by subtracting the initial weight of the scaffolds without TCP (explained in "In vitro scaffold degradation") from the initial weight of the scaffolds with TCP (3 mg). The weight of TCP added to the core-sheath, porous, and single component fibers was quantified as 700µg, 1200µg, and 900µg, respectively. In order to quantify the weight of calcium ions in the scaffolds from this amount of TCP doped within each scaffold, the separate weight of each of the tricalcium phosphate components, i.e. the weights of the orthophosphates and calcium ions, were used. By incorporating the molar mass of calcium ions (Ca^{2+}, 40.08 g/mol), orthophosphates (PO₄³⁻), 94.97 g/mol), and tricalcium phosphate (Ca₃(PO₄)₂, 310.18 g/mol) the weight of Ca^{2+} doped within each scaffold was calculated to be 271 µg for the core-
sheath fibers, 465 µg for the porous fibers, and 349 µg for the single component fibers. The percentage of Ca$^{2+}$ released from each scaffold could then be calculated using Equation 1.

$$\text{Release\%} = \frac{\text{Total weight of Ca}^{2+}\text{doped in scaffold} - \text{Weight of Ca}^{2+}\text{remaining in scaffold}}{\text{Total weight of Ca}^{2+}\text{doped in scaffold}} \times 100 \quad (1)$$

**In vitro scaffold degradation**

Scaffolds removed from HCL were placed under a fume hood for 3 days to ensure complete evaporation of solvent. Their weight was measured for calculation of weight loss percentages of the scaffolds throughout the 36 day experimental duration. To obtain the initial weight of the scaffolds for calculation of weight loss, three control scaffolds (single component, core-sheath, and porous) were also prepared and digested in HCL prior to immersion in CGM and degradation. The primary weight of the pure PLA scaffolds (i.e., TCP content = 0%) was quantified by calculating the mean weight of three samples. The weight loss percentage was then calculated using Equation 2:

$$\text{Scaffold weight loss \%} = \frac{\text{Initial weight of scaffold} - \text{Final weight of scaffold}}{\text{Initial weight of scaffold}} \times 100 \quad (2)$$

**Cell seeding and analysis**

Human adipose derived stem cells (hASC) were obtained from excess human adipose tissue obtained from a voluntary liposuction procedure performed on a 36 year old Caucasian female in accordance with an approved IRB protocol (IRB-04-1622) at University of North Carolina, Chapel Hill. Human ASC were isolated as previously reported by our lab [30-32...]. Human ASC were pre-cultured in complete growth medium (CGM) (Eagle’s Minimum Essential Medium, alpha-modified supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units ml$^{-1}$ penicillin and 100 µg ml$^{-1}$ streptomycin) to 80% confluency in 75 cm$^2$ tissue culture flasks, trypsinized, suspended in
CGM, and seeded on the TCP loaded scaffolds with different fibrous morphologies comprised of single component, porous, or core-sheath fibers at an initial cell seeding density of $2 \times 10^4$ cells/cm$^2$. Scaffolds were kept in osteogenic differentiation medium (ODM) (containing complete growth medium supplemented with 50 μM ascorbic acid, 0.1 μM dexamethasone, and 10 mM β-glycerolphosphate) for 21 days with the media changes every 3 days throughout 21 days of culture. Cell viability was determined with a Live/Dead Assay Cytotoxicity Kit (Molecular Probes, Eugene, OR) at days 1 and 21.

To quantify the hASC-mediated calcium accretion in the scaffolds, scaffolds were washed twice with 1×PBS then soaked in 0.5 N HCl and the supernatant tested using the Calcium Liquicolor Assay (Stanbio, Boerne, TX). In order to determine the cell mediated calcium accretion per cell, the protein content in cells was also quantified using the BCA protein assay (Thermo Fisher Scientific Inc, IL) for normalization purposes.

### 2.3 Results and Discussion

**Scaffold characterization**

*Single component fiber scaffolds*

Single component fibers were successfully loaded with TCP nanoparticles. The fibers had average fiber diameters of 450±72 nm. Surface morphology analysis of the fibers confirmed the presence of TCP on the fibers (Figure 2.1). The average thickness of the fibrous webs was measured and determined to be 30±7 μm.
Figure 2.1: SEM micrographs of single component PLA fibers doped with TCP.

Core-sheath fiber scaffolds

TCP nanoparticles incorporated in PLA were used to create the core polymer in core-sheath fibers. Pure PLA was used as the sheath polymer, effectively enclosing TCP in the core and inhibiting TCP particle formation on the surfaces of the fibers. Scanning electron microscopy analysis indicated fibers had smooth surfaces (Figure 2.2a,b) with an average fiber diameter of 890±125 nm. The average thickness of the electrospun core-sheath fibrous mat was 50±11 µm. Transmission electron microscopy analysis confirmed the existence of TCP inside the core of the core-sheath fibers (Figure 2.2c).

Figure 2.2: SEM (a,b) and TEM (c) micrographs of core sheath PLA fibers.
Porous fiber scaffolds

Porous fibers were created from a solution of PLA in dichloromethane with an average fiber diameter of 1020±164 nm and web thickness of 40±9 μm. The scanning electron microscopy confirmed that the fibers had nano-scale elliptical pores throughout the fibers with an average pore size of 40nm along the major axis and 20nm along the minor axis (Figure 2.3). Pores were uniformly distributed throughout the length of all fibers.

The formation mechanism of the pores was a function of the large temperature difference between the surface of the electrospinning jet and the surrounding media [33, 34]. The solvent used, Dichloromethane (DCM), has a vapor pressure of 47 kPa at 20°C. The fast rate of evaporation resulted in further evaporative cooling of the fiber surface during electrospinning. As the surface of the jet cooled, moisture grew and condensed on the jet. When the solvent evaporated and the electrospinning jet dried, the moisture droplets left pores on the surface of the fibers, known as breath figures [29]. These porous PLA scaffolds were then loaded with 10 wt% TCP (Figure 2.4). SEM micrographs indicated that although addition of TCP changed the uniformity of the fibers and the pores on their surface, pore formation still occurred during the electrospinning process.
Surface area analysis

Surface area of the fibers directly depends on their fiber diameter and surface morphology. Thinner fibers have a higher surface area. Surface porosity of fibers also significantly increases fiber surface area. The BET results indicated a higher surface area for regular nanofibers compared to porous and core-sheath fibers (Table 2.2).

Table 2.2: Surface area of different fiber structures

<table>
<thead>
<tr>
<th>Type</th>
<th>Fiber Diameter (nm)</th>
<th>Multipoint BET (m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single component fibers</td>
<td>450±72</td>
<td>2.181</td>
</tr>
<tr>
<td>Core-sheath fibers</td>
<td>890±125</td>
<td>1.127</td>
</tr>
<tr>
<td>Porous fibers</td>
<td>1020±164</td>
<td>1.541</td>
</tr>
</tbody>
</table>

In vitro release analysis

Analysis of release of TCP from the scaffolds indicated that the initial rate of release for single component and porous fibers exhibited a burst release profile then the release rate of the calcium ions diminished (Figure 2.5). Core-sheath fibers, however, exhibited a distinctly different release profile.
For these fibers the initial rate of release was not as fast as porous and single component fibers. Rather, the rate of release for the core-sheath fibers remained fairly constant during the 36 days of analysis.

Figure 2.5: Release profile of different fiber structures

The highest overall amount of TCP release (approximately 90%) occurred with the porous fibers. Generally three different mechanisms are employed to explain the release of encapsulated compounds from nanofibers: desorption, degradation (for biodegradable polymers) and diffusion. The release rate can be clarified by desorption of the embedded compound from nanopores on the outer surface of the fibers in contact with the surrounding medium. Through the desorption mechanism, only compounds in direct contact with the surrounding medium can be released [35]. Degradation allows for dopants encapsulated within biodegradable polymers to be released gradually as time passes [36]. Polymer degradation occurs via two different mechanisms for polymer degradation. One is surface erosion in which the polymer does not allow water molecules to penetrate into it, thereby allowing for only surface degradation to occur. The second is bulk erosion in which a polymer with hydrophilic
groups has the capability to degrade both from its surface and from within the polymer itself [35]. PLA, the polymer used in this study, exhibits bulk degradation behavior. When PLA degrades its molecular weight reduces by random cleavage of the ester bonds so the byproducts of lactic acid and oligomers form [37].

Release is also a function of diffusion of the compounds. Diffusion results in the movement and transport of atoms in solid phases. Typically this release mechanism has been attributed to solid-state diffusion of an encapsulated compound from the fibers into the surrounding medium [38]. The release of TCP nanoparticles from the different structures of the PLA scaffolds created and analyzed in this study can be described by a combination of these three different mechanisms of release, i.e., a combination of desorption, degradation, and diffusion. However since the diffusion process is extremely slow, it is likely not the dominant mechanism of release in this study which only considers 36 days of release. It is likely that the desorption and degradation are the primary mechanisms for release of the TCP nanoparticles in this investigation. For the single component and porous fibers, the high initial release of TCP was likely due to desorption of the TCP nanoparticles attached to the surfaces of the fibers. With core-sheath fibers in which the TCP nanoparticles are not present on the surface of the scaffolds but rather encapsulated in the core of the fibers, the release is possibly due to the sporadic nanopore formation on the surface of fibers after incubation [39]. Due to the presence of such pores, the surrounding medium is still able to be in direct contact with the core and facilitate drug release from the core by desorption. Degradation of PLA is another process which can assist release of the TCP nanoparticles encapsulated within the core-sheath fibers. Release of the TCP nanoparticles via these two mechanisms requires a greater period of time, therefore the release rate of TCP was slower with core-sheath fibers, and it also slowed after the initial burst release pattern exhibited by the single component and porous fibers. Complete degradation and diffusion did not
occur over 36 day time period of investigation and the release of TCP from the PLA scaffolds was not complete. The greater amount of overall release from the porous fibers was likely due to the presence of nanopores throughout the fibers, increasing desorption of the TCP nanoparticles and enhancing transport of the surrounding medium into the fibers and thus allowing for faster fiber degradation.

**Scaffold degradation analysis**

Biodegradation of the polymers was determined by weight loss as a function of time. Calculating the percentage of weight loss for the three different fibrous structures, it was determined that the porous fibers exhibited the greatest weight loss during the 36 day experimental duration (approximately 30%) ([Figure 2.6](#)). As described above this was likely a result of enhanced diffusion of the medium through the fibers as a result of nanopores on the surfaces of the porous fibers. The minimum weight loss occurred with core-sheath fibers (less than 10%). Considering the release profiles of all three scaffolds, it is appears that degradation of the PLA scaffolds is in direct relation with the release of TCP nanoparticles. The lowest and highest amount of PLA degradation corresponded to core-sheath and porous fibers which have the least and greatest amount of TCP release respectively.

Core sheath nanofibers with their larger fiber diameters (890±125 nm) in comparison to single component fibers (450±72 nm) exhibited less surface area ([Table 2.2](#)), an important factor affecting the degradation rate as greater surface area allows for faster degradation.
Cell viability

Viability analysis of hASC seeded on the TCP loaded scaffolds created using single component, porous or core-sheath fibers illustrated that after 1 and 21 days, cells were well attached to the surfaces of the scaffolds in a random orientation (Figure 7). Live-dead staining indicated that only a small number of cells were not viable, the majority of hASC were viable and well spread across the scaffold surface.
Figure 2.7: Viability images of human adipose derived stem cells (hASC) seeded on TCP-loaded electrospun scaffolds comprised of single component (a, d), porous (b, e), or core-sheath (c, f) fibers. Green = live cells; red = dead cells.

Calcium quantification results (Figure 2.8) confirmed osteogenic differentiation of hASC in all scaffolds at days 7, 14 and 21. Moreover, these results indicated higher calcium accretion per cell for hASC seeded on porous fibers as compared to single component and core-sheath fiber scaffolds.
2.4 Conclusions

Electrospun PLA scaffolds having different fiber morphologies, single component, porous and core-sheath, were loaded with TCP nanoparticles for investigation of the release profiles of these different fiber morphologies for future potential use in bone tissue engineering applications. The release profile of TCP from each of the different structures was analyzed over 36 days. The results indicated that core-sheath fibers exhibited a relatively steady release behavior while porous fibers exhibited the highest initial burst release. Therefore, core sheath fibers have great potential for encapsulating compounds that are best administered via a sustained release profile. Weight loss analysis of the three different structures indicated that the highest amount of degradation occurred with porous fibers. Live/dead analysis indicated that all three fiber morphologies maintained high hASC viability with only minimal cell death. Human ASC osteogenic differentiation varied for the three scaffolds. In particular hASC seeded on porous fibers exhibited highest osteogenic differentiation and the least osteogenic differentiation was seen for core-sheath fibers. This is the first study to investigate control of the release rate of TCP nanoparticles from a scaffold and the first to determine effects of these differential releases on hASC viability and osteogenic differentiation. The findings hold great
potential for bone tissue engineering applications. Our results suggest that the morphology of fibers in electrospun scaffolds is an essential parameter in controlling the release profile of calcium ions in the culture medium, which can ultimately influence the osteogenic differentiation, and cell-mediated bone regeneration of stem cells, osteoblasts, and osteoblast precursors.

2.5 Acknowledgement

The authors would like to thank the Nonwovens Cooperative Research Center for funding (Project 10-128, EGL); and all other members of the Cell Mechanics Laboratory.
REFERENCES


24. A. K. Moghe, B. S. Gupta, Polymer Reviews, **2008**, *48*, 353


Chapter 3

Novel, Silver Ion Releasing Nanofibrous Scaffolds Exhibit In Vitro and In Vivo Antibacterial Efficacy without the Use of Silver Nanoparticles

Submitted to Acta Biomaterialia, August 2013

Nanofibers, with their morphological similarities to the extracellular matrix of skin, hold great potential for skin tissue engineering. Over the last decade, silver nanoparticles have been extensively investigated in wound healing applications for their ability to provide antimicrobial benefits to nanofibrous scaffolds. However, the use of silver nanoparticles has raised concern as these particles can penetrate into the stratum corneum of skin, or even diffuse into the cellular plasma membrane. We present and evaluate, both in vitro and in vivo, a new silver ion release polymeric coating that we have found can be applied to biocompatible, biodegradable poly (L-lactic acid) (PLA) nanofibrous scaffolds. Using this compound, custom antimicrobial silver ion releasing nanofibers were created. The presence of a uniform, continuous silver coating on the nanofibrous scaffolds was verified using X-ray photoelectron spectroscopy (XPS) analysis. Antimicrobial efficacy of the antimicrobial scaffolds against Staphylococcus aureus and Escherichia coli bacteria was determined in vitro via industry-standard AATCC protocols. Cytotoxicity analyses of the antimicrobial
scaffolds towards human epidermal keratinocytes and human dermal fibroblasts were performed via quantitative analyses of cell viability and proliferation. Our results indicated the custom antimicrobial scaffolds exhibited excellent antimicrobial properties while also maintaining human skin cell viability and proliferation for silver ion concentrations below 62.5 µg/ml within the coating solution. Antimicrobial scaffolds that supported human skin cell growth and proliferation were then evaluated in vivo in a porcine model. Our findings confirmed that the antimicrobial scaffolds were capable of killing and inhibiting the growth of bacteria in both open and closed wounds infected with Staphylococcus pseudintermedius bacteria. This is the first study to show that silver ions can be effectively delivered with nanofibrous scaffolds to an infected wound site without the use of silver nanoparticles.
3.1 Introduction

Although minor skin wounds can heal naturally, immediate coverage using skin substitutes is necessary for treatment of extensive or irreversible damages caused to skin (1). An ideal skin substitute should protect the wound from microorganism contamination and further physical damage while also accelerating the healing process and minimizing scarring (2). Many xenograft (3), allograft (4,5), and autograft (6,7) skin substitutes have been investigated for wound healing. However, due to limited donor sites from which to acquire autografts, risk of infection, slow healing, and scar formation associated with these approaches, creation of tissue engineered skin substitutes has become an active area of investigation (8). Many advancements in tissue engineered skin substitutes have been achieved in the past three decades using natural or synthetic polymeric scaffolds (9,10). From these studies, it is now understood that the polymeric materials used should meet specific requirements related to hemostatic and anti-adhesion properties, air and moisture permeability, and appropriate absorption properties such that extraneous body fluid is extracted while the wound bed is kept moist to prevent dehydration (11). Nanofibers created from biodegradable, biocompatible polymers encompassing these properties are an excellent option for skin tissue engineering. Nanofibers are morphologically similar to the native extracellular matrix (ECM) of skin and have been shown to promote cell adhesion, migration and proliferation, leading to faster healing (2,12).

As a result of the beneficial outcome achieved with the use of nanofibers for normal skin healing, they have been recently explored for their ability to incorporate antimicrobial agents in an attempt to prevent infection at a wound site. Silver is a well-known
antimicrobial compound that is highly toxic to a wide range of microorganisms (13). It is an FDA-approved (14) broad-spectrum biocide that kills over 650 disease-causing bacteria, fungi, viruses, and mold (13). There is no life-threatening risk caused by inhalation, ingestion, or dermal application (15). If silver penetrates into the human body, it enters the systemic circulation as a protein complex, and can be eliminated by the liver and kidneys (15). Therefore it is not dangerous to humans when delivered in the proper chemical form and concentration (15). Although silver is relatively inert, its interaction with moisture coming from the surface of the skin and with fluids in the wound bed leads to the release of silver ions. Silver ions are highly reactive and bind to bacterial DNA and RNA, denaturing them resulting in bacterial growth inhibition (16).

The key to optimizing the use of silver as an antimicrobial agent is to maximize the production of silver ions that can be achieved by increasing the surface area of metallic silver. Silver in the form of nanoparticles has been incorporated in nanofibers by either in situ generation of nanoparticles within the polymer (17-19) or dispersion of prepared nanoparticles in the system (ex situ synthesis) (20,21). However, the very small size of nanoparticles makes them able to penetrate into the stratum corneum of skin (22,23) or diffuse within the cellular plasma membrane and interfere with a variety of cellular mechanisms (21,24). For this reason, in recent years, the FDA and others (25-27) have recently expressed concern with antimicrobial approaches incorporating silver nanoparticles.

New methodologies and approaches to harness the excellent antimicrobial properties of silver ions, without the use of nanoparticles, need to be actively explored. In this study, we proposed and investigated delivery of silver ions using silver nitrate with a suitable
polymeric binder. We hypothesized that the polymeric binder would adhere to the surface of biocompatible, biodegradable poly(L-lactic acid) (PLA) nanofibers, provide an insoluble coating when water and ethanol were evaporated from the polymeric binder, and release silver ions capable of killing a variety of clinically relevant bacteria both in vitro and in vivo. We further hypothesized that an appropriate concentration of silver release could be achieved using this system to simultaneously kill and inhibit growth of bacteria while maintaining viability and proliferation of human skin cells.

### 3.2 Materials and methods

*Nanofibrous scaffold fabrication*

Polylactic acid (PLA), a biodegradable, biocompatible polymer with a molecular weight of 70000g/mol was dissolved in chloroform and DMF (both obtained from Sigma, St Louis, MO, USA) at a ratio of 3 to 1 to create a 12% solution. The mixture was stirred on a magnetic stirrer plate for at least 4 hours at 80°C until a homogeneous solution was obtained. Polymer solutions were used within 24 hours of preparation to eliminate evaporative loss of solvent and consequent change in solution concentration. The PLA solution was electrospun using 15 kV voltage, feed rate of 0.7 µl/hr and spinning distance of 13-15 cm. Silvadur ET (Dow Chemical Company, Midland, MI, USA) solutions were made and diluted to various concentrations to coat the PLA nanofibers. Specifically, the initial concentration of silver in Silvadur ET is 1.0 mg/ml. This solution was diluted in deionized water to reduce the amount of silver within the solution to concentrations of 500, 250, 125, 62.5 and 31.25 µg/ml. Nanofibers were dipped in the diluted Silvadur ET solution for one hour and then dried under
a fume hood for 24 hours to form a thin antimicrobial coating. Coated nanofibers were then sterilized with ethylene oxide gas for 12 hours.

Scaffold characterization

The thickness of the scaffolds was measured with a Mitutoyo absolute micrometer (Aurora, IL). Average thickness from at least 30 measurements was calculated. Field emission scanning electron microscopy (FESEM JEOL 6400 F) was used to characterize surface morphology and microstructure of the ultra fine electrospun fibers at 15 kV and 200 kV accelerating voltage, respectively. Using SEM images, average fiber diameter of the scaffolds was calculated from at least 50 measurements per scaffold. To confirm the presence of the silver coating, X-ray photoelectron spectroscopy (XPS) was performed using a Kratos analytical axis ultra spectrometer (Chestnut Ridge, NY) for at least three different locations of both non-coated PLA scaffolds and Silvadur ET coated samples.

Release profiles of silver ions from PLA scaffolds

Scaffolds were soaked in deionized water and maintained in an incubator at 37°C and 5% CO₂ for two weeks. At specific time points: 24, 48, 84, 120 and 324 hours, the water was removed and replaced with fresh deionized water. The concentration of silver ions released in deionized water at each time point was quantified using a Perkin-Elmer AA300 atomic adsorption spectrophotometer (AAS) (PerkinElmer Inc. Waltham, MA).

Antimicrobial efficacy of scaffolds

Antimicrobial properties of the scaffolds were assessed against both gram negative (Escherichia coli J53) and gram positive (Staphylococcus aureus) bacteria as well as a silver (Ag) resistant bacteria (Escherichia coli J53 [pMG101]) as a control to ensure that the
The antimicrobial properties of the scaffolds were a result of silver content alone. Cation-adjusted Mueller-Hinton (MH) broth and agar (Difco Laboratories, Detroit, MI, USA) were used to prepare bacterial cultivating medium. Isolated bacterial colonies were grown overnight in an incubator (37°C, 5% CO₂) from frozen samples on an agar plate. For Ag-resistant bacteria, 100 mg/ml ampicillin sodium salt (Fisher Scientific, USA) was also added to the agar plates. Antimicrobial activity of the scaffolds against different bacteria was determined by both a) qualitative evaluation, Antibacterial Activity Assessment of Textile Materials – AATCC 147, and b) quantitative evaluation, Assessment Of Antimicrobial Finishes on Textile Materials - AATCC 100.

**Qualitative analysis of the antimicrobial activity of the scaffolds: AATCC 147**

For this analysis (parallel streak method), scaffolds were cut into circles (d=1.6 cm), placed on a lawn of bacteria in an MH agar plate, and incubated overnight at 37°C. Each lawn was prepared by spreading a 0.5 McFarland suspension of bacteria in phosphate buffered saline (PBS) using a sterile swab. The inoculants used included Staphylococcus aureus, Escherichia coli J53 and Escherichia coli J53 [pMG101]. Bactericidal activity was visually assessed to determine the zone of inhibition around the fiber mat after overnight incubation per the AATCC 147 protocol to evaluate antimicrobial efficacy of the scaffolds against the inoculated bacterium.

**Quantitative analysis of the antimicrobial activity of the scaffolds: AATCC 100**

For quantitative evaluation, scaffolds were cut into 1cm² squares and soaked in 2 ml MH broth inoculated with Escherichia coli or Staphylococcus aureus bacteria at an initial concentration of 10⁶ CFU/mL. To control the accuracy of bacterial density, bacteria were
diluted in PBS at $10^3$, $10^4$, $10^5$, and $10^6$ CFU/mL and plated overnight. Suspensions were then incubated at 37°C for one week. Bacterial concentration after incubation was determined via serial dilution of the suspension and spreading 100 µL of each diluent on a tissue culture plate. Evenly spread bacteria on plates allowed for the isolation of individual colonies for quantification after overnight incubation. Non-treated PLA scaffolds were used as negative controls. Equation 1 (per AATCC 100 protocol) was used to calculate the reduction% as follows:

$$\text{Reduction}\% = 100(B-A)/B$$

where A is the number of bacteria recovered from the inoculated antimicrobial PLA scaffold after one week; and B is the number of bacteria recovered from the inoculated uncoated, control PLA scaffold. 0% reduction indicates no antimicrobial efficacy; 100% reduction indicates complete antimicrobial efficacy.

### 3.3 Biocompatibility assessment of scaffolds

Biocompatibility assessment of antimicrobial scaffolds was performed using human dermal fibroblasts and human epidermal keratinocytes derived from adult skin (2nd passage) purchased from Lonza (USA). Nanofibers coated with Silvadur ET containing different concentrations of silver: 250, 125, 62.5, 31.25 or 15.75 µg/ml, were cut into circles (d=1.6 cm). Sterilized samples were soaked in fibroblast or keratinocyte growth medium (Lonza, USA), respectively cell type being evaluated, for 24 hr and then seeded with cells at an initial density of $4 \times 10^4$ cells per scaffold. Cell-seeded scaffolds were then maintained in growth medium for 7 days at 37°C and 5% CO2. Culture medium was replaced every three days.
Human skin cell viability

Cell viability was determined on days 1, 4 and 7 using a Live-Dead Assay Cytotoxicity Kit for mammalian cells (Molecular Probes, Eugene, OR). Specifically, cell seeded scaffolds were put in growth medium with the addition of 4 mM calceinacetoxyethyl ester-AM (staining the cytoplasm of live cells green) and 4 mM ethidiumhomodimer (staining the nuclei of dead cells red). The samples were incubated for 20 minutes while protected from light.

Human skin cell proliferation

Cell proliferation was determined using the alamarBlue cell viability assay (AbDSerotec, Raleigh, NC) at days 1, 4 and 7 post seeding of cells on scaffolds. AlamarBlue, at a volume of 10% of the culture medium, was added to each well 7 hours before each measurement. After incubation with alamarBlue, 200 µL of each sample was taken in triplicate and the absorbency read at 600 nm using a microplate reader (TecanGENios, Tecan, Switzerland). AlamarBlue reduction was calculated from the absorbency results using the manufacturer’s protocol. A greater alamarBlue reduction indicates greater cell proliferation.

Human skin cell morphology

Scanning Electron Microscopy (SEM) was used to visualize fibroblast and keratinocyte morphologies on the scaffolds. After 7 days of culture in growth medium, samples were fixed in 10% buffered formalin for 30 min and then dehydrated with a graded concentration (50-100% v/v) of ethanol. Dehydrated scaffolds were immersed in
hexamethyldisilazane for 15 min and dried overnight under a fume hood. Dried samples were sputter coated with gold to observe the morphology of cells using SEM.

3.4 In vivo evaluation of antimicrobial scaffolds

All animal procedures were approved by the Institutional Animal Care and Use Committees (IACUC) at North Carolina State University. An incision wound model was implemented in a porcine model to assess the performance of antimicrobial scaffolds in vivo. Antimicrobial scaffolds were coated with solution containing 62.5 µg/ml silver. Scaffolds were cut into squares of 3×4 cm². Linear full thickness skin incisions were made along the back of five 45-50kg female pigs (Yorkshire crosses), each incision measuring 4 centimeters in length (Figure 3.1). Scaffolds were placed inside the wounds subdermally and $10^2$ methicillin-resistant Staphylococcus pseudintermedius (CM-001) bacteria were injected in a portion of the wound sites using a cross-over design (Table 3.1), before suturing the wounds closed. Two uncontaminated incisions were left open (Table 3.1) to also track environmental bacterial infection. One animal was sacrificed at each of the following postoperative time periods: days zero (immediately after surgery), 1, 3, 5, and 7.
Figure 3.1: Open and closed linear full thickness skin incisions on the back of a pig after placement of a scaffold under dermis layer

Table 3.1: Treatment protocol at each wound site (8 wounds/pig on each of 5 pigs, n=5) *

<table>
<thead>
<tr>
<th>Wound</th>
<th>Scaffold</th>
<th>Staph pseud</th>
<th>Open/Closed</th>
<th># on each pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>No</td>
<td>Open</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>No</td>
<td>Closed</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Antimicrobial Scaffold*</td>
<td>No</td>
<td>Closed</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Control PLA Scaffold</td>
<td>No</td>
<td>Open</td>
<td>1</td>
</tr>
<tr>
<td>5, 6</td>
<td>None</td>
<td>Yes</td>
<td>Closed</td>
<td>2</td>
</tr>
<tr>
<td>7, 8</td>
<td>Antimicrobial Scaffold</td>
<td>Yes</td>
<td>Closed</td>
<td>2</td>
</tr>
</tbody>
</table>

*Antimicrobial scaffolds are PLA nanofibers (control scaffolds) coated with solution containing 62.5 µg/ml silver

For bacteriological analyses, an 8mm cylindrical punch biopsy of tissue (radius = 4 mm; height = 5 mm) was taken from each wound site. Volumes were normalized to weight to determine CFU/g of bacteria. Biopsied tissue was homogenized in 9 mL of PBS. One milliliter of each homogenate was placed into a 96-well plate. One hundred microliters of
the homogenate solution was serially diluted into 900 mL of PBS and 100 uL from each dilution plated in triplicate onto CNA agar. Plates were incubated at 35°C overnight. After incubation, all three plates from the dilution were counted and recorded. Those colonies morphologically resembling the inoculated organisms were counted as determined by a clinical molecular biologist. Final concentration was determined based on an average of the three-plate colony count, and the weight of the tissue sample. Inflammation of wounds was qualitatively evaluated by an expert blinded observer (board certified veterinary surgeon (ACVS-SA and ECVS) with over 10 years of experience assessing wounds and incisions for inflammation and infection).

3.5 Statistical analyses

Statistical analyses were performed using SPSS 14.0. The data was analyzed using a Duncan test with p-values less than 0.05 considered statistically significant.

3.6 Results and discussion

Scaffold characterization

SEM images indicated nanofibers were uniformly coated with Silvadur ET and had an average fiber diameter of 460.53 ± 180 nm (Figure 3.2). The average thickness of the nanofibrous webs was 30±7 µm.
XPS indicated silver was present along the entire surface of nanofibers coated with the silver containing solution (Figure 3.3). In addition to silver, oxygen and nitrogen associated with nitrate (in silver nitrate: AgNO₃) were also uniformly distributed along the surface of all nanofibers. Presence of a carbon group as shown in the XPS analysis is also a component of the polymeric binder in the Silvadur ET coating.
Figure 3.3: XPS spectra for PLA nanofibers coated with Silvadur ET (carbon and oxygen are components of PLA, nitrogen and silver are components of Silvadur ET)

Release profile of silver ions from PLA scaffolds

Release profiles of silver ions from antimicrobial scaffolds coated with different concentrations of silver within the coating solution were determined. Results indicated a fast initial release of silver ions, followed by a gradual release of silver over two weeks (Figure 3.4). The release rate was directly influenced by the initial amount of silver doped within the scaffolds. The highest release rate, and amount, occurred with nanofibers coated with solution containing 125µg/ml silver and the lowest release was observed with scaffolds coated with solution containing 15.75 µg/ml silver.
Figure 3.4: Release profile of silver from PLA scaffolds coated with Silvadur ET containing different concentrations of silver varying from 15.75 µg/ml (bottom) to 125 µg/ml (top).

Antimicrobial efficacy of scaffolds

Qualitative analyses of the antimicrobial properties of the custom scaffolds against E. coli and Staphylococcus aureus bacteria indicated clear zones of inhibition around all scaffolds with silver coating (Figure 3.5). No zone of inhibition was observed against silver resistant bacteria indicating that the antimicrobial properties of the scaffolds were specifically a result of silver ion release. The zone of inhibition was measured for all scaffolds treated with coatings containing different concentrations of silver. There was no significant difference between the zones of inhibition for scaffolds coated with different silver concentrations (Figure 3.6).
Figure 3.5: Antimicrobial properties of scaffolds treated with Silvadur ET containing 32.25µg/ml silver on E. Coli (a), Staphylococcus aureus (b), and silver resistant E. Coli (c) bacteria as evaluated via AATCC 147 test.

![Image of Petri dishes showing zones of inhibition]

Figure 3.6: Qualitative antimicrobial test (AATCC 147) for scaffolds coated with different concentrations of Silvadur ET. Columns with different letters are significantly different (p<0.05)

Quantitative antimicrobial analyses (AATCC 100) were performed for one week on scaffolds with varying silver concentrations in the coating. Results indicated 100% reduction of both E. Coli and Staphylococcus aureus bacteria for all scaffolds. In addition to quantification via the AATCC 100 protocol, antimicrobial efficacy was further confirmed visually (Figure 3.7).
Figure 3.7: Quantitative antimicrobial assessment (AATCC 100) of scaffolds coated with Silvadur ET containing 250 µg/ml silver in Silvadur ET coating solution. Cloudy color indicates bacterial contamination; clear color indicates lack of bacteria. 100% reduction was achieved for Silvadur ET coated scaffolds using equation (1).

3.7 Biocompatibility assessment of scaffolds

*Viability of human dermal fibroblasts on antimicrobial scaffolds*

Viability of human dermal fibroblasts grown on the antimicrobial scaffolds was assessed on days 1, 4 and 7. Results indicated that cells exhibited limited or no viability on scaffolds with coatings containing silver concentrations of 125 µg/ml to 250 µg/ml silver in the coating solution (data not shown). However, the majority of cells remained viable on nanofibers with coatings containing silver concentrations of 62.5 µg/ml and 31.25 µg/ml silver (Figure 8). Cells adhered to both coated and non-coated scaffolds and, in both cases, better adhesion of cells was achieved with extended culture duration (Figure 3.8).
Figure 3.8: Viability of human dermal fibroblasts on control (no silver, pure PLA alone) and antimicrobial (31.25 µg/mL silver in Silvadur ET coating of PLA fibers) scaffolds on days 1, 4 and 7

**Viability of human epidermal keratinocytes on scaffolds**

Human epidermal keratinocyte viability was tested only on scaffolds that supported fibroblast viability (silver concentrations in coating solution: 62.5 µg/ml and 31.25 µg/ml) along with an additional scaffold coated with a lower concentration of silver, 15.75 µg/ml. Human epidermal keratinocytes remained viable on all three of these scaffolds. Representative images of human epidermal keratinocytes on scaffolds with 31.25 µg/ml silver in the coating solution (Figure 3.9) were consistent for concentrations of 15.75 µg/ml and 62.5 µg/ml.
Figure 3.9: Viability of human epidermal keratinocytes on control (no silver, pure PLA alone) and antimicrobial (31.25 µg/mL silver in Silvadur ET coating on PLA fibers) scaffolds on days 1, 4 and 7

Proliferation of fibroblasts on antimicrobial scaffolds

Human dermal fibroblast proliferation was diminished on PLA scaffolds coated with higher silver concentrations (Figure 10). AlamarBlue reduction%, an indicator of cell proliferation, remained close to constant for 7 days for scaffolds coated with the most concentrated silver solution (250 and 125 µg/ml silver), indicating no cell proliferation on these scaffolds. These results were consistent with findings from the viability analyses, which indicated no viable cells on scaffolds with these silver concentrations. However, there was no significant difference in cell proliferation on scaffolds coated with the less concentrated silver solution (31.25 µg/ml) relative to uncoated, pure PLA controls at any time point (Figure 3.10).
Figure 3.10: AlamarBlue reduction for human dermal fibroblasts seeded on different scaffolds indicated that 31.25 ug/ml Silvadur ET concentration did not affect cell proliferation relative to 0 ug/ml control. Columns with different letters are significantly different (p<0.05)

*Proliferation of human epidermal keratinocytes on the scaffolds*

Proliferation of keratinocytes on the antimicrobial scaffolds followed the same trend as fibroblasts and was decreased by an increase in silver concentration within the coating solution (Figure 3.11).
Figure 3.11: AlamarBlue reduction for human epidermal keratinocytes seeded on scaffolds containing different silver concentrations indicated that 31.25 ug/ml Silvadur ET concentration did not affect cell proliferation relative to 0 ug/ml control. Columns with different letters are significantly different (p<0.05)

Morphology of cells on the scaffolds

SEM micrographs indicated that fibroblasts adhered and spread on the scaffolds (Figure 12). Fibroblasts spread throughout the surface of uncoated, PLA controls as well as PLA nanofibers coated with solutions containing 31.25 and 62.5 µg/ml silver. However, for nanofibrous scaffolds coated with solutions containing 125 and 250 µg/ml silver, cells exhibited a rounded morphology, indicative of dead cells. These observations were consistent with the viability findings (Figure 3.12).
Figure 3.12: SEM micrographs of human dermal fibroblasts seeded on pure PLA scaffolds (a, b, c), PLA antimicrobial scaffolds coated with Silvadur ET containing 31.25 µg/ml (d, e, f), 62.5 µg/ml (g, h, i), 125 µg/ml (j, k, l) and 250 µg/ml (m, n, p) silver at different magnifications of 100X, 500X, and 2000X (magnification increasing from left to right).
SEM analyses of human epidermal keratinocytes on antimicrobial scaffolds with silver solutions containing 15.75 µg/ml, 31.25 µg/ml, and 62.5 µg/ml silver indicated cells adhered and spread on the scaffolds (Figure 3.13).

Figure 3.13: SEM micrographs of human epidermal keratinocytes on antimicrobial scaffolds coated with Silvadur ET containing 15.75 µg/ml (a, c), 32.25 µg/ml (b, d), and 62.5 µg/ml (c, e) at different magnifications of 500X, and 2000X (magnifications increasing from top to bottom).

3.8 *In vivo* evaluation of antimicrobial scaffolds

Antimicrobial efficacy against methicillin-resistant *Staphylococcus pseudintermedius* was evaluated *in vivo* using a porcine model. No *Staphylococcus pseudintermedius* bacterial growth was observed in non-inoculated open or closed wounds (Table 3.2). In closed
wounds inoculated with Staphylococcus bacteria without antimicrobial scaffolds, an increase in the number of bacteria from day 0 (no detectable bacteria) to day 1 (20.60 CFU/g) and day 3 (595.33 CFU/g) was observed. Day 3 had the highest population of bacteria in the wound site. However, after day 3, bacterial concentrations diminished in all wound sites indicating that the animals were able to clear the infections naturally by day 5. This was not unexpected given these animals had normal immune systems. In an immune compromised patient, it is probable that bacterial growth could continue to the level of severe infection, consistent with the trend observed from days 0 – 3.

Wounds treated with antimicrobial scaffolds had significantly less bacteria at these time points (day 1: 3.83 CFU/g in antimicrobial scaffold vs. 20.60 CFU/g in control; day 3: 0 CFU/g for antimicrobial vs. 595.33 CFU/g in control) (Table 2). The highest bacterial count in wounds treated with antimicrobial scaffolds was more than 10 fold less than the highest bacterial count in control wounds (21.25 CFU/g at day 5 in treated wounds vs. 595.33 CFU/g at day 3 in control wounds), indicating that the antimicrobial scaffolds were capable of inhibiting bacterial growth in vivo.
Table 3.2: Staphylococcus pseudintermedius concentrations in control and treated wounds*

<table>
<thead>
<tr>
<th>Wounds</th>
<th>Staphylococcus pseudintermedius bacterial concentration (CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>1. Control open wounds</td>
<td>---</td>
</tr>
<tr>
<td>2. Control closed wound</td>
<td>---</td>
</tr>
<tr>
<td>3. Closed wound + scaffold</td>
<td>---</td>
</tr>
<tr>
<td>4. Open wound + scaffold</td>
<td>---</td>
</tr>
<tr>
<td>5. Closed wound + Bacteria</td>
<td>---</td>
</tr>
<tr>
<td>6. Closed wound + Bacteria + scaffold</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* See Table 1 for description of wound condition. Dashes (---) indicate bacterial growth negligible as evaluated by a blinded, clinical molecular biologist.

Values for inflammation scoring range from 0 (no inflammation) to 5 (severe inflammation).

There was no major inflammation (inflammation score >3) in any of the wounds (Table 3.3), as scored by a blinded, expert veterinary observer. The highest inflammation scores for all wounds occurred on day 3; the same time point where the greatest bacterial concentration was found.
Table 3.3: Inflammation scoring of control and treated wounds*

<table>
<thead>
<tr>
<th>Wounds</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control open wounds</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2. Control closed wound</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3. Closed wound + scaffold</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4. Open wound + scaffold</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5. Closed wound + Bacteria</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>6. Closed wound + Bacteria + scaffold</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Refer to Table 1 for explanation of treatment protocol for each wound site.

Inflammation scores range from 0 (no inflammation) to 5 (severe inflammation).

Images of closed and open wounds at day 3 for both control and antimicrobial treated wounds indicated tissue in control, untreated wounds was red, inflamed, and exhibited greater purulent discharge relative to wounds treated with antimicrobial scaffolds (Figure 3.14).
Figure 3.14: Closed (a,c) and open (b, d) wounds inoculated with Staphylococcus bacteria with (a,b) and without (c,d) antimicrobial scaffolds at day 3

3.9 Conclusions

Silver ion releasing PLA nanofibrous scaffolds were developed using a silver ion releasing solution (Silvadur ET) comprised of silver nitrate, a proprietary polymer binder, water and ethanol. Multiple silver concentrations were created and evaluated as a coating solution on PLA nanofibers for antimicrobial efficacy \textit{in vitro} and \textit{in vivo}. Nanofibers coated with solutions containing different concentrations of silver ranging from 31.25 to 250 µg/ml exhibited excellent antimicrobial properties toward both gram positive and gram negative bacteria while having no effect on silver resistant bacteria. This verification against silver resistant bacteria was performed to confirm that antimicrobial properties of the scaffolds were a result of silver content alone. Biocompatibility analyses of the antimicrobial scaffolds indicated that nanofibrous scaffolds coated with silver solutions containing silver equal to or
less than 62.5 µg/ml maintained viability and proliferation of both human dermal fibroblasts and human epidermal keratinocytes. Optimized scaffolds were then further validated in vivo using a porcine model. In vivo results indicated that no major inflammation was induced by the antimicrobial scaffolds; and further confirmed the ability of such scaffolds to inhibit the growth of clinically relevant bacteria in a highly infected wound site. This is the first study to use a polymeric coating containing silver nitrate on nanofibrous scaffolds. These novel antimicrobial scaffolds are the first nanofibrous substrates that contain silver in a form other than nanoparticles to effectively address concerns related to the use of silver nanoparticles while still exhibiting excellent antimicrobial efficacy. Our in vitro and in vivo experiments confirmed that silver can be used in nanofibrous bandages for wound healing applications if delivered in the right form and concentration. We believe the clear-cut process for development of these antimicrobial scaffolds allows for facile scale-up of these bandages with multiple applications in wound healing to allow for skin regeneration while maintaining antimicrobial efficacy.

3.10 Acknowledgements

This research was supported by NIH/NIBIB 1R03EB008790 (EGL), NSF/CBET 1133427 (EGL), Nonwovens Cooperative Research Center (Project 10-128, EGL), and Chancellor’s Innovation Fund (EGL). The authors would like to acknowledge Dr. Kyle Mathews for his help with the in vivo experiments and Dr. Susan Bernacki for assistance with the in vitro experiment; also all other members of the Cell Mechanics Laboratory.
REFERENCES


Chapter 4

Skin Tissue Engineering for the Infected Wound Site: Biodegradable PLA Nanofibers and a Novel Approach for Silver Ion Release Evaluated in a Co-Culture System of Keratinocytes and Staphylococcus aureus

Submitted to Tissue Engineering Part A, July 2013

Wound infection presents a challenging and growing problem. With the increased prevalence and growth of multi drug-resistant bacteria, there is mounting need to reduce and eliminate wound infection using methodologies that limit the ability of bacteria to evolve into further drug-resistant strains. A well-known strategy for combating bacterial infection and preventing wound sepsis is via delivery of silver ions to the wound site. High surface area silver nanoparticles allowing extensive silver ion release have therefore been explored in different wound dressings and/or skin substitutes. However, it has been recently shown that nanoparticles can penetrate into the stratum corneum of skin or diffuse into the cellular plasma membrane, and interfere with a variety of cellular mechanisms. The goal of this study was to introduce and evaluate a new type of high surface area metallic silver in the form of highly porous silver microparticles. PLA nanofibers were successfully loaded with either highly porous silver microparticles or silver nanoparticles and antimicrobial efficacy and cytotoxicity of the two silver-based wound dressings assessed and compared. To better
mimic the physiological environment \textit{in vivo} where both human cells and bacteria are present, a novel co-culture system combining human epidermal keratinocytes and \textit{Staphylococcus aureus} (\textit{S. aureus}) bacteria was designed to simultaneously evaluate human skin cell cytotoxicity with antimicrobial efficacy. We found that highly porous silver microparticles could be successfully incorporated in nanofibrous wound dressings, and exhibited comparable antimicrobial efficacy and cytotoxicity to silver nanoparticles. Further, PLA nanofibers containing highly porous silver microparticles exhibited steady silver ion release, at a greater rate of release, than nanofibers containing silver nanoparticles. Replacement of silver nanoparticles with the newly introduced microparticles overcome concerns regarding the use of nanoparticles and hold great promise as skin substitutes or wound dressings for infected wound sites.
4.1 Introduction

Wound infection presents a challenging and growing problem, in particular for those individuals with compromised immune systems. Wound infection delays wound closure (1, 2), diminishes tensile strength of the healing wound tissue, increases length of hospital stay and costs, and increases the patient’s risk of bacteremia, sepsis, multisystem organ failure, and death (3). With the increased prevalence and growth of multi drug-resistant bacteria, there is mounting need to reduce and eliminate wound infection using methodologies that limit the ability of bacteria to evolve into further drug-resistant strains. A well-known strategy for combating bacterial infection and preventing wound sepsis is via delivery of silver ions to the wound site (4). Silver ions can be introduced to infected wound sites by a number of different mechanisms. A well known technique is utilization of silver salt solutions, the most effective of which is silver nitrate (AgNO₃) (5). Aqueous silver nitrate at a concentration of 0.5% has been shown effective as a topical treatment for burn patients(5). Silver nitrate is usually applied to the wound site by wetting cotton gauze dressings with silver nitrate solution every 3-4 hours per day(6). Other forms of silver have been investigated, in particular silver sulfadiazine. Silver sulfadiazine is also delivered topically to burn wounds, typically in a 1% cream or suspension(7). Silver sulfadiazine provides a sufficient concentration of silver and its residual activity is higher than silver nitrate, allowing for application only twice per day.

While both these approaches have proven successful for their antimicrobial and antibacterial attributes, they lack the ability for continued release of silver ions, requiring
constant re-application of the compound to painful wound sites. The large, rapid release of silver ions from silver nitrate and silver sulfadiazine can also have negative effects on the host cells, as explained later in this article. Therefore, interest has grown in methods to deliver silver ions continuously, without constant re-application of the silver-containing compound. A recent technique to address this challenge is to incorporate metallic silver in wound dressings(8). When the wound dressings are exposed to fluids in the wound bed and tissue exudates, a sustained release of silver ions occurs (9). The total amount of silver ions that can be released from metallic silver depends on the available surface area (10, 11). High surface area silver nanoparticles allowing extensive silver ion release have therefore been explored in different wound dressings and/or skin substitutes(8). Their excellent antimicrobial efficacy has been shown, however, concern has grown regarding the release of silver nanoparticles from different products into the environment. It is now known that nanoparticles can penetrate into the stratum corneum of skin(12, 13) or even diffuse into the cellular plasma membrane, and interfere with a variety of cellular mechanisms (14, 15). The ability to harness the antimicrobial benefits of high surface area metallic silver in wound dressings, without the use of nanoparticles, remains a significant challenge. A solution has yet to be determined.

When implementing silver in wound dressings, the cytotoxicity of the dressings to the host cells is as important a component of their fabrication as their antimicrobial properties. Although it has been traditionally reported that silver causes low mammalian cell toxicity (16, 17), silver ions do have cytotoxic effects on some mammalian cells. It has been shown that epidermal keratinocytes and dermal fibroblasts, the two dominant cell types in human
skin, are susceptible to lethal damage when exposed to high concentrations of silver (18). However, since cytotoxicity is directly proportional to silver concentration (19), this problem can potentially be addressed by using metallic silver dressings with controlled release capabilities, allowing for complete wound healing to occur (20). Ultimately, the desired goal is to achieve an optimized release profile that results in antimicrobial/antibacterial efficacy without mammalian cell cytotoxicity (20).

The goal of this study was to introduce and evaluate a new type of high surface area metallic silver in the form of highly porous silver microparticles. The microparticles are composed of agglomerated silver nanoparticles, resulting in the desired high surface area without undesired detachment and release of nanoparticles in the environment. These high surface area microparticles were incorporated within polylactic acid (PLA) nanofibrous dressings. Biodegradable, biocompatible PLA nanofibers, with their morphological similarities to the extracellular matrix of skin, have great potential to be used as skin substitutes and wound dressings (21). PLA nanofibers were loaded with either highly porous silver microparticles or silver nanoparticles and antimicrobial efficacy and cytotoxicity of the two silver based wound dressings assessed and compared. To better mimic the physiological environment in vivo where both human cells and bacteria are present, a novel co-culture system combining human epidermal keratinocytes and Staphylococcus aureus (S. aureus) bacteria was designed to simultaneously evaluate cytotoxicity and antimicrobial efficacy. This new co-culture system could also be used to evaluate any other antimicrobial dressings.
4.2 Materials and methods

Scaffold fabrication

Polylactic acid (molecular weight = 70000g/mol) was dissolved in chloroform and DMF (Sigma, St Louis, MO, USA) at a ratio of 3 to 1 to create a 12% solution. Silver nanoparticles (20 nm Citrate Biopure™Silver) (Nanocomposix Company, San Diego, CA, USA) or highly porous silver microparticles (average diameter = 6.9 µm, surface area = 3.9m²/g) (BioGate Company, Nuremberg, Germany) (Figure 4.1) were added to the PLA solution to obtain a 0.5% concentration silver to polymer ratio. Mixtures were stirred on a magnetic stirrer plate for 4 hours at 80°C then sonicated for 30 minutes to further ensure particle dispersion. Polymer solutions were used immediately after sonication to eliminate particle precipitation (of particular importance for microparticles) and prevent evaporative loss of solvent and consequent change in solution concentration. The PLA solution was electrospun in a custom electrospinning system (22) for two hours using 15 kV voltage, feedrate of 0.7 µl/hr and spinning distance of 13-15 cm. Electrospun scaffolds were kept under a fume hood overnight to fully evaporate residual solvents. Scaffolds were then removed from the fiber collector and cut into circles using a punch (d=1.6 cm). Scaffolds were sterilized with ethanol for 10 minutes then rinsed three times with phosphate buffered saline (PBS) and soaked in keratinocyte growth media (KGM-Gold, Lonza, USA) without antibiotics for 12 hours to allow proteins to attach to the scaffolds. Pure PLA scaffolds containing no silver were used as controls.
Figure 4.1: SEM micrograph of the surface of highly porous silver microparticles

Silver release studies

Scaffolds were soaked in deionized water and incubated at 37°C and 5% CO₂ for one week. At specific time points: 3, 6, 18, 30, 42, 66, 120 and 168 hours, half of the water was removed and replaced with fresh deionized water. The concentration of silver ions released at each time point was quantified using the removed water via a Perkin-Elmer AA300 atomic adsorption spectrophotometer (AAS) (PerkinElmer Inc. Waltham, MA).

Co-culture system of human epidermal keratinocytes with S. aureus

To evaluate the antimicrobial efficacy of silver nano- or microparticle loaded scaffolds when seeded with cells, a co-culture system and experimental process was designed (Figure 4.2). Separate experiments with only human epidermal keratinocytes or S. aureus bacteria were also performed to better understand and elucidate the results of the co-culture system (Table 4.1). PLA scaffolds loaded with silver microparticles, silver
nanoparticles, or neither (pure PLA, controls) were seeded with human epidermal keratinocytes derived from adult skin (2nd passage, Lonza, USA) at a density of $4 \times 10^4$ cells per scaffold and incubated for 24 hours. The cell seeded scaffolds were then inoculated with 10 CFU/ml S. aureus bacteria (AATCC# 43300™) dispersed in keratinocyte growth media without antibiotics. Epidermal keratinocyte- and S. aureus-seeded scaffolds were then placed and maintained in an incubator (37 °C, 5% CO$_2$) for 72 hours on a rotating plate, assisting with the suspension of bacteria.

Figure 4.2: Co-culture system to evaluate human skin and/or other mammalian cells in combination with bacteria on three-dimensional nanofibrous scaffolds
Table 4.1: Design of experiment for antimicrobial and cytotoxicity evaluation of scaffolds either separately or in a co-culture system

<table>
<thead>
<tr>
<th></th>
<th>Scaffold</th>
<th>Replicates</th>
<th>Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytotoxicity experiment</strong></td>
<td>Pure PLA</td>
<td>3</td>
<td>AlamarBlue assay</td>
</tr>
<tr>
<td></td>
<td>PLA+Ag Nanoparticles</td>
<td>3</td>
<td>DNA assay</td>
</tr>
<tr>
<td></td>
<td>PLA+Ag Microparticles</td>
<td>3</td>
<td>DNA assay</td>
</tr>
<tr>
<td><strong>Antimicrobial experiment</strong></td>
<td>Pure PLA</td>
<td>3</td>
<td>Spectrophotometry</td>
</tr>
<tr>
<td></td>
<td>PLA+Ag Nanoparticles</td>
<td>3</td>
<td>Plate count</td>
</tr>
<tr>
<td></td>
<td>PLA+Ag Microparticles</td>
<td>3</td>
<td>Plate count</td>
</tr>
<tr>
<td><strong>Co-culture experiment</strong></td>
<td>Pure PLA</td>
<td>3</td>
<td>Spectrophotometry</td>
</tr>
<tr>
<td></td>
<td>PLA+Ag Nanoparticles</td>
<td>3</td>
<td>DNA assay</td>
</tr>
<tr>
<td></td>
<td>PLA+Ag Microparticles</td>
<td>3</td>
<td>DNA assay</td>
</tr>
</tbody>
</table>

**Monitoring S. aureus growth in co-culture system**

At specific time points (12, 24, 26, 42, and 72 hours), 100 µl of culture medium was taken (after pipetting up and down several times to ensure bacteria were well suspended in the medium), diluted multiple times, and spread on Muller-Hinton (MH) agar plates (Thermo Scientific, USA). Agar plates were incubated overnight to allow bacterial colonies to grow and become visible to the naked eye for counting and monitoring bacterial growth in each well. After 72 hours, bacterial counts were confirmed by measurement of the optical density of the bacterial suspension, taken from each sample, using a UV-vis spectrophotometer (Biomate3, Thermo Electron Corporation, Madison, WI, USA) at a wavelength of 600 nm.

**Human epidermal keratinocyte viability and proliferation in co-culture system**

Viability analyses were performed at day 1 (24 hours after keratinocyte seeding on the scaffolds and prior to addition of bacteria) and day 3 (72 hours after addition of bacteria) using a fluorescent method (Live-Dead Assay Cytotoxicity Kit for mammalian cells; Molecular Probes, Eugene, OR). Specifically, keratinocyte-seeded scaffolds were placed in KGM with the addition of 4 mMcalceinacetoxymethyl ester-AM (staining the cytoplasm of
live cells green) and 4 mMethidiumhomodimer (staining the nuclei of dead cells red). The samples were then incubated for 20 minutes while protected from light. Cell proliferation, in cultures without bacteria, was determined with a cell viability assay (alamarBlue, AbDSerotec, Raleigh, NC) at different time points after seeding of cells on scaffolds (days 1, 2, and 3). AlamarBlue, at a volume of 10% of the culture medium, was added to each well 7 hours before each measurement. After incubation with alamarBlue, 200 µL of each sample was taken in triplicate and the absorbency read at 600 nm using a microplate reader (TecanGENios, Tecan, Switzerland). Greater alamarBlue reduction% indicated greater cell proliferation. Since AlamarBlue assay is not an appropriate method to evaluate the proliferation of cells in co-culture systems (presence of bacteria changes the reduction%), the number of cells on the scaffolds was quantified by measuring the DNA content in each scaffold after 72 hours. The scaffolds were washed at least three times with PBS to confirm that the bacteria were detached from the scaffolds. To verify that all bacteria were washed out from the scaffolds, the PBS solution from the last wash was used for bacterial analysis to confirm no bacteria were present in PBS from the last wash. Once this was confirmed, the amount of DNA in each nanofibrous scaffold was then measured with DNA binding dye Hoechst 33258 in microplate format after an overnight digestion at 60°C in 2.5 units/mL papain in PBS with 5mM Ethylenediaminetetraacetic acid and 5mM cysteine HCl (all reagents from Sigma).

**Microscopic analyses**

Keratinocyte morphology on the scaffolds was evaluated using scanning electron microscopy (SEM) (FESEM JEOL 6400 F) at 15 kVaccelerating voltage. At each time
point, nanofibrous scaffolds were fixed in 10% buffered formalin for 30 min and then dehydrated with a graded concentration (50-100% v/v) of ethanol. Dehydrated scaffolds were immersed in hexamethyldisilazane for 15 min and dried overnight in a fume hood. Dried samples were sputter coated with gold to observe the morphology of cells using SEM. Transmission electron microscopy (TEM) (Hitachi HF2000) was used to further characterize longitudinal dispersion of particles within ultra fine electrospun fibers at 200 kV accelerating voltage.

**Statistical analyses**

Statistical analyses were performed using SPSS 14.0. Data were analyzed using Duncan test with p-values less than 0.05 considered statistically significant.

### 4.3 Results

Electrospun PLA fibers containing either silver nanoparticles or highly porous microparticles formed in a uniform manner on the fiber collector (Figure 4.3(a,d)). However, as expected, incorporation of the two particles within the fibers varied. SEM images indicated that silver nanoparticles were not present on the surface of fibers and there were very few locations where nanoparticles were close to the fiber surface (Figure 4.3(b)). TEM analysis confirmed the presence of well-dispersed nanoparticles inside the fibers, closer to the core (Figure 4.3(c)).

Highly porous silver microparticles on the other hand, were too large to be encapsulated inside the nanofibers. SEM (Figure 4.3(e)) and TEM (Figure 4.3(f)) images showed that these particles were present on the surface of fibers.
Release profiles revealed that silver ions gradually release from both scaffolds, doped with silver nanoparticles or highly porous silver microparticles, over a one week period (Figure 4.4). Silver ion release was slow at the first two time points (3 and 6 hours) for both scaffolds, then significantly accelerated at the 16 hour time point for nanofibers doped with highly porous silver microparticles (Figure 4). Rate of release and cumulative release remained greater for scaffolds doped with highly porous silver microparticles at all remaining time points.
AlamarBlue findings indicated that proliferation of keratinocytes was diminished on PLA scaffolds loaded with silver nano- or microparticles compared to pure PLA scaffolds, with lowest proliferation observed for silver nanoparticle loaded scaffolds (Figure 4.5). Similarly, viability analyses indicated that neither silver nano- or microparticle loaded scaffolds supported human epidermal keratinocyte viability after three days (Figure 4.6). Addition of bacteria to the culture medium further decreased cell viability for all three keratinocyte-seeded scaffolds (Figure 4.6).
Figure 4.5: Human epidermal keratinocyte proliferation on scaffolds without bacteria in the culture medium. Different letters indicate significant difference (p-value < 0.05).
SEM micrographs showed that keratinocytes adhered and spread throughout the pure PLA scaffolds. However, keratinocyte morphology was clearly different on PLA scaffolds loaded with silver nano- or microparticles. On those scaffolds, cell number was reduced and cells were rounded, representing morphology of dead cells (Figure 4.7). Consistent with qualitative viability analyses (Figure 4.6), addition of bacteria to the culture medium further reduced the number of viable cells on pure PLA scaffolds. SEM images also confirmed presence of S. aureus bacteria with rounded morphology on the all three scaffolds.
DNA quantitation (Figure 4.8) indicated that pure PLA scaffolds supported the greatest keratinocyte viability relative to either silver nano- or microparticle loaded scaffolds in either the presence or absence of bacteria, consistent with viability (Figure 4.6) and SEM (Figure 4.7) analyses. DNA content significantly dropped for pure PLA scaffolds with the addition of bacteria to the culture medium.
Figure 4.8: Human epidermal keratinocyte DNA quantitation on scaffolds both in the presence and absence of S. aureus bacteria. Different letters indicate significant difference between groups (p-value < 0.05).

Bacterial analyses indicated that S. aureus proliferated on all three scaffolds, with the highest rate of growth for pure PLA scaffolds without keratinocytes (Figure 4.9). These results not only indicate that addition of silver nano- or microparticles to the PLA scaffolds can reduce bacterial growth rate, but also that the presence of keratinocytes in co-culture systems with S. aureus diminishes bacterial growth.
4.4 Discussion

In this study, a new metallic silver compound comprised of highly porous silver microparticles was successfully incorporated within polylactic acid electrospun nanofibers using a conventional electrospinning approach. Gradual, sustained release of silver ions from these scaffolds was observed over a seven day experimental period. Interestingly, the rate of silver ion release was significantly higher for silver microparticle loaded scaffolds relative to silver nanoparticle loaded scaffolds. This can be related to the different cross sectional locations of the two particles within the fibers. Dopants closer to the outer layer of fibers release faster than those well incorporated inside the core (23). Silver nanoparticles located inside the fiber therefore release silver ions slower than highly porous silver microparticles that, by function of their size, are present at the surface of fibers. Cytotoxicity analyses of the silver nano- and microparticle loaded scaffolds indicated that keratinocytes do not
proliferate as well on either of these scaffolds as on pure PLA scaffolds. Our findings are consistent with previous investigators who have reported diminished proliferation of keratinocytes in the presence of silver ions (24-26).

As expected, antimicrobial analyses revealed a delay in the growth of *S. aureus* bacteria on silver ion release scaffolds relative to pure PLA controls. However, the amount of silver ions released from the two scaffolds was not adequate to kill all bacteria within the solution with an initial concentration of 10 CFU/mL *S. aureus* bacteria that was evaluated. Although increasing the initial concentration of silver particles in nanofibers can increase the release of silver ions (23) and increase their antimicrobial properties, we would not recommend such an approach except for severely infected wounds given the cytotoxic effects of increased silver concentration on human epidermal keratinocytes that we found. The rate of bacterial growth over 72 hours was not significantly different for scaffolds containing micro or nanoparticles of silver. This finding was not unexpected given that the maximum difference in silver ion release between the two scaffolds was small (maximum difference = 0.042 µg/ml, 66 hour time point) and not likely to result in a differential antibacterial response.

To avoid complex interactions between mammalian cells and bacteria, the majority of previous studies have evaluated the antimicrobial and cytotoxicity of antimicrobial agents separately. Such an approach may not appropriately address the *in vivo* environment. To address this some recent studies have tried to design co-culture systems of cells and bacteria to test antimicrobial agents that can be added to the culture medium (27-30). To date, there is no published method on evaluating antimicrobial dressings in co-culture systems.
Therefore, we designed a novel co-culture system to assess cytotoxicity and antimicrobial properties of antimicrobial scaffolds in one experiment. Our results confirmed that antimicrobial and cytotoxic performance of silver containing scaffolds alters when both bacteria and human skin cells are present in one culture system. The presence of *S. aureus* reduced the viability and proliferation of human epidermal keratinocytes on all three scaffolds. This has been reported previously by other researchers who merely studied the interaction of cells and bacteria in the absence of dressings (30, 31). In addition, the growth of *S. aureus* bacteria was diminished on scaffolds seeded with epidermal keratinocytes relative to acellular scaffolds. This phenomenon is likely attributed to release of antimicrobial peptides when the bacteria come in contact with the keratinocytes (32, 33).

In conclusion, we found that highly porous silver microparticles could be successfully incorporated in nanofibrous wound dressings, and exhibited comparable antimicrobial efficacy and cytotoxicity to silver nanoparticles. Further, PLA nanofibers containing highly porous silver microparticles exhibited steady silver ion release, at a greater rate of release, than nanofibers containing silver nanoparticles. Replacement of silver nanoparticles with the newly introduced microparticles overcome concerns regarding the use of nanoparticles and hold great promise as skin substitutes or wound dressings for infected wound sites.
4.5 Acknowledgements

This research was supported by NIH/NIBIB 1R03EB008790 (EGL), NSF/CBET 1133427 (EGL), Nonwovens Cooperative Research Center (Project 10-128, EGL), and Chancellor’s Innovation Fund (EGL). The authors would like to acknowledge Dr. Susan Bernacki for helping with the bacterial experiment; also all other members of the Cell Mechanics Laboratory.
REFERENCES


Chapter 5

Ibuprofen Loaded PLA Scaffolds for Wound Healing Applications

Submitted to Biomaterials, October 2013

This paper investigates the controlled delivery of ibuprofen through an electrospun poly (L-lactic acid) (PLA) nanofibrous bandage for wound healing purposes. The long term goal of such work is the treatment of chronic wounds in vivo to provide a continued release of an anti-inflammatory compound without cytotoxic effects on host skin cells. Three different weight percentages of ibuprofen were loaded into electrospun PLA scaffolds at concentrations of 10, 20 and 30 wt% by polymer weight. The chemical integrity of ibuprofen after exposure to the high voltage source required for electrospinning was analyzed and confirmed by $^1$H NMR spectroscopy. The release rate of ibuprofen from the bandages into deionized water was quantified at either room temperature or 37°C. As expected, the release of ibuprofen was faster for bandages with higher initial concentrations of ibuprofen at higher environmental temperature, 37°C. In vitro evaluation of ibuprofen loaded bandages indicated human epidermal keratinocytes remained viable and proliferated on bandages loaded with all ibuprofen concentrations with the highest proliferation for 20 wt% ibuprofen-doped PLA scaffolds. Scaffolds doped with 20% ibuprofen were further evaluated in vivo for wound healing applications using a full thickness skin incision model in nude mice. In
*vivo* findings indicated that wound contracture is diminished for wounds covered with ibuprofen loaded bandages relative to non-treatment controls. However, histological and immunohistological analyses indicated that skin regeneration may be delayed with use of acellular bandages alone. The bandages were then seeded with human dermal fibroblasts and epidermal keratinocytes. Ibuprofen-loaded bandages seeded with human dermal fibroblasts and human epidermal keratinocytes exhibited significantly less wound contracture with significantly enhanced skin regeneration relative to non-treatment controls.
5.1 Introduction

The largest population of chronic wounds is comprised of venous leg ulcers, diabetic foot ulcers, and pressure ulcers. As of 2007, approximately 10 billion dollars was spent in the United States alone to treat these chronic wounds [1]. In addition to ulcer patients, burn patients can also develop chronic wounds. Approximately 100,000 people in the US suffer from burn injuries in a year ranging from moderate to severe [2]. Inflammatory responses prolong the healing process. If prolonged localized inflammation is not treated, the patient can develop systemic inflammation, potentially resulting in septic shock [2]. Patients of severe burns require extensive wound healing treatments in order to recover; and, in most cases the connective tissue is not as strong as the original tissue[3].

Inflammation is prolonged if inflammatory responses are not treated. Acute inflammation is a short-term response that usually results in healing; however, chronic inflammation results in tissue damage (Campbell, 2008). The numbers of neutrophils decrease, replaced by macrophages in the latter stage of inflammation [4]. Although macrophages are initially favored, the persistence of macrophage activity can lead to chronic inflammation and thus chronic wounds. Inflammation can also cause pain and immobility of the patient, hindering tissue repair [5]. Therefore, decreasing inflammation in chronic wounds would be favorable in wound healing. Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) and analgesic that is a more cost effective alternative to other NSAIDs such as Naproxen (Hamilton, 2010).
Another important aspect of wound healing that needs to be considered is the type of wound dressing to be utilized. Many properties of wound dressings are key to successful tissue regeneration. They include, but are not limited to, non-toxicity, carrier for anti-septics, sterilizability, conformability, and minimum replacement [6]. The need for the first four of these properties is obvious. Minimal replacement is not as obvious a requirement but also critical as it has been shown that dressings that need to be changed frequently disturb the wound bed and delay healing [6]. Therefore, an attractive alternative to most materials used for current wound dressings is a dressing that is both biocompatible and biodegradable allowing for efficacious wound healing without, or with minimal, bandage changes. As a result, investigations of the use of electrospun bioresorbable polymers for wound healing applications have grown. Nanofibrous bandages produced by electrospinning are morphologically similar to the cells’ extracellular matrix, allowing cells to adhere and proliferate. (Travis J. Sill, 2008). Electrospinning also permits doping of drugs into the scaffold fibers, providing the potential to develop a controlled drug release system.

Poly(L-lactic acid) (PLA) is a FDA approved, biocompatible, and bioresorbable polymer with strong mechanical properties [7]. The by-product of PLA is lactic acid, which can be easily absorbed by the body [8]. Previous investigations have shown that poly (lactic-co-glycolic acid) (PLGA) at multiple ratios of PLA and PGA can be doped with ibuprofen for drug delivery from this biodegradable, biocompatible scaffold/bandage material. PLGA scaffolds containing 10% by polymer weight of ibuprofen exhibited complete degradation within only 6 days [9]. PLA and PGA are of great interest for controlled delivery systems due to their hydrolytic degradation [8].
The mechanical properties of PLA are stronger than that of PLGA and therefore take longer to degrade [7]. Given the economic cost of treating chronic wounds, it would be beneficial to lower the cost of the dressing. In this regard, PLA is an attractive economical alternative to PLGA. Therefore, the aim of this study was to develop a controlled release system of ibuprofen via ibuprofen incorporation and controlled release from a PLA nanofibrous bandage. Ibuprofen release profiles from PLA nanofibrous bandages containing 10, 20 or 30 wt% ibuprofen were determined and cytotoxic effects on human epidermal keratinocytes (HEK) evaluated \textit{in vitro}.

\section*{5.2 Materials and methods}

\textit{Scaffold development}

Poly(L-lactic acid) (PLA) (Gift from Nonwovens Cooperative Research Center) was dissolved in dimethylformamide (DMF) and chloroform in a 1:3 ratio. (S)-(+) Ibuprofen (Sigma-Aldrich, St. Louis, MO), an enantiomeric form of ibuprofen (Figure 5.1) used for pharmokinetic studies (Jamali F, 1992), was added to the PLA solution in DMF and chloroform at concentrations of 10, 20 and 30 wt% ibuprofen relative to polymer weight. Two ml of the ibuprofen polymer solution were drawn into a 10 ml syringe. A needle with an internal diameter of 0.508mm was attached to the end of the syringe. The syringe contents were electrospun using a syringe pump at a constant feed rate of 0.2 ml/hr for 1 hour. Electrospinning was performed in a horizontal orientation with a high voltage source attached to the grounded collector plate as previously described [10, 11]. A 12-15kV range was delivered to the collector and the needle to draw the polymer solution out of the syringe and to a grounded collector plate. The scaffold was produced at room temperature at a
humidity of approximately 50%. Scaffolds were punched into circles (diameter = 1 cm) and weighed. Punched, circular scaffolds were placed into a Harrick Plasma Cleaner PDC- 32 G (Harrick Plasma). The scaffolds were plasma treated at atmospheric conditions for 30 seconds on each side. Scaffolds were then dried under a fume hood for 24 hours prior to sterilization with ethylene oxide.

![Chemical formula of (S)-(+) Ibuprofen](image)

2Figure 5.1: Chemical formula of (S)-(+) Ibuprofen

### 5.3 Scaffold characterization

*Scanning electron microscopy*

A scanning electron microscope (SEM, JEOL JSM-6400F) was used to determine fiber morphology. Revolution software (JEOL JSM-6400F) was used to measure individual fiber diameters. 45 samples of fiber diameters were taken from two 10000X images from each of the four different scaffold types, pure PLA, 10, 20 and 30% ibuprofen loaded PLA scaffold.

*Nuclear Magnetic Resonance (NMR) spectroscopy*
$^1$H NMR spectra were recorded using a 500 MHz Bruker DRX spectrometer operating at 500.128 MHz proton frequency with Topspin 1.3 software version (Bruker, Karlsruhe, Germany). All spectra were collected with 90° $^1$H pulse of 10.75 µs, relaxation delay of
2s, and 128 scans. The NMR signal was Fourier transformed without apodization and the phase and baseline carefully corrected. TMS (tetramethylsilane) was used as an internal standard. Assignments of NMR signals to the corresponding chemical compounds ibuprofen and PLA were made by using prediction software ACD (Advance Chemistry Development) (Toronto, Ontario, Canada) and confirmed by previous 2D NMR experiments and $^{13}$C spectra. Samples were prepared for NMR spectroscopy by dissolving each sample in approximately 0.6 ml of deuterated chloroform (CDCl$_3$) and transferring the solution to a 5-mm NMR tube for analysis. Tubes were carefully washed and dried for 24 hours in an oven and bubbled with nitrogen before being capped for storage. The NMR probe was tuned to $^{13}$C frequency, i.e., 125.75 MHz in the 500 MHz spectrometer ($^1$H frequency -500.128 MHz). Data was processed with Bruker software Topspin 1.3 and standard processing parameters.

*Release of ibuprofen from nanofibrous PLA bandages*

Ibuprofen-loaded (10, 20, or 30 wt% ibuprofen) or pure (0 wt% ibuprofen) PLA bandages were placed separately in 0.5ml of sterilized deionized water and maintained at either room temperature or 37°C (body temperature). Fluid samples were removed from each bandage-containing solution at 2, 4, 8, 10, 24, 48, 96, 144, 192, 240, 288 and 336 hours and stored in centrifuge tubes at room temperature covered in aluminum foil. All samples were measured within 24 hours of their respective time points to avoid evaporative loss. The 0.5ml of deionized water was replaced after each time point. Prior to UV/Vis spectroscopy analyses, 0.5mL of 100% ethanol was added to dissolve any insoluble ibuprofen particles.
2.5µL of the solution was inserted on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and measured at an intensity of 220nm and 218 nm for all time points. The results were compared to a calculated standard curve. Three replicates of each bandage were measured and used to calculate average release over time for each time point for two weeks.

5.4 **In vitro analyses with human epidermal keratinocytes**

*Culture of adult human epidermal keratinocytes*

Normal adult human epidermal keratinocytes (HEK), passage 2, were purchased from Lonza and immediately frozen. Keratinocyte growth medium (KGM) was created using a KGM-Gold and BulletKit (Lonza) per manufacturer’s protocol. Scaffolds were soaked in KGM for 24 hours in a 12 well plate. After 24 hours, HEK, frozen at passage 2, were thawed, pelleted and resuspended in 875µl of KGM. 70µl of HEK/KGM solution was pipetted directly onto each bandage to obtain a 40k density within each well.

*Viability and proliferation of keratinocytes on bandages*

The viability of the HEKs on pure PLA or ibuprofen-loaded PLA nanofibrous bandages was analyzed using a Live-Dead Assay Cytotoxicity Kit for mammalian cells (Molecular Probes, Eugene, OR). Specifically, cell seeded scaffolds were put in growth medium with the addition of 4 mM calceinacetoxymethyl ester-AM (staining the cytoplasm of live cells green) and 4 mM ethidiumhomodimer (staining the nuclei of dead cells red). The samples were incubated for 20 minutes while protected from light then analyzed using a fluorescent microscope (Leica Microsystems, Buffalo Grove, IL). Proliferation of cells on both PLA control and ibuprofen loaded PLA bandages were analyzed using an alamarBlue
assay (Life Technologies, Grand Island, NY) and UV/Vis spectrophotometry to determine metabolic activity of the cells.

5.5  *In vivo evaluation of 20% ibuprofen loaded bandages*

*Wound contraction*

Since the highest proliferation of keratinocytes was achieved on bandages with 20wt% ibuprofen, these bandages were prepared for an *in vivo* experiment using a nude mice model. In this experiment, a 2 cm×2 cm full-thickness incision wound, which is considered as a large skin injury, was created on the back of 9 mice using sharp scissors while they are anesthetized. Three of the wounds, fall in the control category, were covered with a transparent film (Tegaderm ™, 3M) and a regular cotton bandage that was stitched to the mice skin. Two layers of 2×2 cm² pre-fabricated scaffolds were placed on the wounds of three other mice and covered the same way as control wounds. For the last three mice, instead of two layers of acellular scaffolds, cell seeded scaffolds (top layer seeded with human epidermal keratinocytes and bottom layer seeded with human dermal fibroblasts (Lonza, USA)) were placed on the wounds. For all 9 mice, the external cotton bandage was changed at days 4, 7 and 10 before day 14. At each bandage change, the wound was photographed and inspected for signs of infection or abnormal inflammation. Contraction of wounds was quantified by applying the following formula to the wound photographs.

\[
\text{Contraction} \% = \frac{(A-B)}{A} \times 100 \quad \text{Equation (1)}
\]

where A is the area of the initial wound and B is the remaining wound area after a specific time period. These values were achieved by analyzing images using Fiji software.
Histological analyses

Harvested skin samples were wrapped around a needle and maintained in paraformaldehyde for 24 hours to fix the tissue. Samples were then submerged in a solution of 30% sucrose in phosphate buffered saline (PBS) for 24 hours. The skin samples were then cut in the center into two pieces. One piece of each sample was embedded in paraffin for histological analyses and the other piece was frozen for immunohistochemistry analyses. Paraffin-embedded skin rolls and bandages that were detached from the wounds were sectioned to 5µm thick samples. The samples were then dehydrated and stained using hematoxylin and eosin stains. Hematoxylin stains the nuclei of cells in blue and eosin colors the cytoplasm and extracellular matrix in varying degrees of pink. Thickness of the regenerated skin and the number of blood vessels formed in the wounded area were quantified using hematoxylin and eosin stained images using Fiji software.

Immunohistological analyses

Frozen tissue blocks were cut into 6 µm thick slices with acryostat. Antigen retrieval was carried out by microwaving the cryostat sections in citrate buffer (pH 6) and cooling to room temperature. For staining with Ki 67 and keratin 10, tissue sections were permeabilized with 1% TritonX-100 in 1X PBS and nonspecific proteins were blocked using a 1% bovine serum albumin (BSA) in 1X PBS at room temperature for 15 minutes. Next, slides were incubated for one hour at room temperature with primary antibodies, Keratin 10 antibody (Covance PRB-159P) and Ki-67 antibody (Dako M7249), both diluted in 1:200 in antibody diluents. Subsequently, slides were washed with PBS and incubated at room temperature with secondary antibodies, alexa fluor 594 goat anti-rat IgG and alexa fluor 488
goat anti-rabbit IgG (both from Invitrogen, USA) for an hour in the dark. Slides were then were washed with PBS and covered with DAPI 1:300 in PBS for 5 minutes. Finally, tissue sections were washed and mounted with prolong gold then visualized with a fluorescence microscope (Leica Microsystems, Buffalo Grove, IL).

Statistical analyses

Statistical analyses were performed using SPSS 14.0. The data was analyzed using a Duncan test with p-values less than 0.05 considered statistically significant.

5.6 Results

Scaffold morphology and characterization

SEM images (Figure 5.2) confirmed ibuprofen-loaded bandages maintained nanofibrous morphology with minimal beading, indicating that the ibuprofen/PLA solutions were homogenous when they were electrospun. Fiber diameter was measured using SEM images and indicated that as the percent weight of ibuprofen loaded into PLA increases, the fiber diameter increases (Table 5.1).

Figure 5.2: SEM images at 5000X magnification of ibuprofen loaded PLA bandages in weight percent of (a) 10%, (b) 20%, and (c) 30% ibuprofen. Scale bar = 5μm
Table 5.1: Fiber diameter of 10, 20 and 30wt% ibuprofen loaded PLA bandages

<table>
<thead>
<tr>
<th>Ibuprofen% in scaffold</th>
<th>Diameter (nm)</th>
<th>Weight (mg/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>329.11 ± 249.62</td>
<td>0.428</td>
</tr>
<tr>
<td>20%</td>
<td>478.31 ± 167.61</td>
<td>0.67</td>
</tr>
<tr>
<td>30%</td>
<td>585.38 ± 131.51</td>
<td>0.69</td>
</tr>
</tbody>
</table>

The NMR spectra (Figure 3) displayed an increased peak height respective to an increased percent weight of ibuprofen. Additionally, the labeled peaks indicated that the chemical integrity of ibuprofen was maintained in all three samples, as indicated by the consistency of the ibuprofen peak locations and splitting patterns (Figure 5.3).

Figure 5.3: H NMR spectra from top to bottom of pure PLA, 10, 20, 30 weight percent ibuprofen, and pure ibuprofen labeled with unique PLA (P) and Ibuprofen peaks (I)
Controlled release of ibuprofen

Two trends were observed when analyzing the ibuprofen release profiles. First, an increased temperature (37°C) caused a greater release from the ibuprofen loaded PLA bandages as compared to room temperature (Figure 4). Second, the 30% ibuprofen loaded PLA scaffolds at 37°C produced the highest release of approximately 0.25 mg at 336 hours (Figure 5.4). For both temperature conditions, room temperature and 37°C, the data suggested that a direct correlation existed between the ibuprofen concentration in the bandages and the amount of ibuprofen released.

Figure 5.4: Controlled release of ibuprofen loaded PLA bandages at both room temperature and 37°C (body temperature)
In vitro analysis

AlamarBlue results suggested that the ibuprofen loaded PLA bandages resulted in greater human epidermal keratinocyte proliferation than pure PLA. The 20wt% ibuprofen bandages, in particular, resulted in the highest amount of proliferation in two weeks. However, interestingly, HEK seeded on 30wt% ibuprofen bandages exhibited the lowest amount of metabolic activity among the ibuprofen loaded PLA bandages (Figure 5.5). The metabolic activity was significantly different at day 14 between pure PLA (0 wt%) controls and 10 and 20wt% ibuprofen loaded bandages. Additionally, the 20wt% ibuprofen loaded bandage resulted in significantly different HEK proliferation than both the 10 and 30wt% ibuprofen loaded bandages at day 14. 20wt% ibuprofen loaded bandages were not significantly different than 10wt% ibuprofen loaded scaffolds; however both the 10 and 20% ibuprofen loaded scaffolds were significantly different from the 30% loaded scaffold (Figure 5.5).

Figure 5.5: AlamarBlue assay results for control PLA and 10, 20 and 30% ibuprofen loaded PLA bandages over a 14 day experimental duration –bars with different letters represent significant difference (p < 0.05).
Live/dead images (Figure 5.6) confirmed alamarBlue findings, showing that the 30wt% ibuprofen scaffold had a greater amount of dead cells present at 14 days relative to both the 10 and 20wt% ibuprofen loaded scaffolds. The 20% ibuprofen loaded scaffold contained the highest concentration of live cells at 14 days. By day 14, the control, pure PLA scaffolds exhibited the least number of viable, proliferating cells (Figures 5.5 and 5.6).

![Figure 5.6: Live/dead images of HEK on PLA control and 10, 20 and 30% ibuprofen loaded PLA scaffolds.](image)

**Visual evaluation of wounds**

Visual evaluation of control wounds receiving no treatment (Figure 5.7, top row) indicates that natural wound healing over 14 days occurs with extensive contraction of existing skin toward the wounded area while new skin is regenerating on the wound site. Comparing control and scaffold-treated wounds at day 14, it is visually noticeable that this contraction is less for all wounds treated with scaffolds (cell seeded or non-seeded scaffolds) (Figure 5.7).
Wound contraction was further quantitatively evaluated (Equation 1) and the results confirmed a trend of lower contraction values for both scaffold treated wounds, with the acellular scaffolds resulting in significantly less contraction relative to the untreated control (Figure 5.8). Images from the wounds treated with scaffolds also exhibited appropriate degradation of scaffolds over time, causing the scaffolds to break apart (Figure 5.7, middle row).
Figure 5.8: Contraction of wounds with/without scaffolds (acellular or cell seeded) - bars with different letters represent a significant difference

Histological analyses

Cross sections of scaffolds stained with hematoxylin and eosin showed that skin cells could migrate through the pores of both layers of the scaffolds over the two week experimental period (Figure 5.9). Hematoxylin and eosin staining of skin samples harvested from the wound site clearly showed distinct differences in morphology of the regenerated skin as compared to native, unwounded skin in all samples (Figure 5.10). Both skin thickness and the number of blood vessels of newly regenerated skin were quantified using these images (Table 5.2). Results indicated that the thickness of regenerated skin was less for the scaffold treated wounds and the smallest thickness was related to the wounds treated with acellular scaffolds. Blood vessel count results indicated a lower number of blood vessels formed on wounds treated with acellular scaffolds. However, there was no significant difference in blood vessel density between wounds covered with cell-seeded scaffolds and untreated control wounds (Table 5.2).
Figure 5.9: Hematoxylin and eosin staining of cell seeded scaffold 14 days after placement on wound site – cells are present on both the surface of scaffolds as well as migrated through the pores.

Figure 5.10: Hematoxylin and eosin staining of harvested skin samples from control wound sites without scaffolds (a,d), with acellular scaffold (b,e) and with HEK seeded scaffold (c, f) – the bottom pictures are the magnified areas in yellow rectangular in top pictures.
Table 5.2: Regenerated skin thickness and number of blood vessels formed

<table>
<thead>
<tr>
<th>Wound coverage</th>
<th>Number of mice</th>
<th>Thickness of regenerated skin</th>
<th>Number of blood vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>No scaffold</td>
<td>3</td>
<td>923±94</td>
<td>53.5</td>
</tr>
<tr>
<td>Acellular scaffold</td>
<td>3</td>
<td>774±162</td>
<td>30.5</td>
</tr>
<tr>
<td>Cell seeded scaffold</td>
<td>3</td>
<td>801±129</td>
<td>55.0</td>
</tr>
</tbody>
</table>

**Immunohistochemistry analyses**

Keratin 10 and Ki-67 were used as markers of differentiating and proliferating cells in harvested skin samples. Keratin 10 is a differentiating product of keratinocytes and expression of keratin 10 demonstrate the formation of epidermal layer. In the regenerated skin on control, untreated wound sites, formation of an epidermis layer was clearly observed (Figure 5.11(a)). Acellular-scaffold treated wounds did not exhibit an epidermal layer in most regions of regenerated skin. Reduced expression of keratin 10 in cell seeded-scaffold treated wounds was also observed. Ki-67-immunopositive cells were observed predominantly in the basal layer and hair follicles of healthy skin. In regenerated skin, these actively cycling cells were also observed with higher population on the scaffold-treated wounds (Figure 5.11).
Figure 5.11: Representative images of immunofluorescent stained regenerated skin on wounds with no scaffold (a), with acellular scaffold (b), or with cell seeded scaffold (c) – keratin 10 is stained epidermis in green and Ki 67 stained proliferating cells in pink (arrows)

5.7 Discussion

In this study, we successfully loaded ibuprofen into a biodegradable PLA scaffold. PLA has strong mechanical properties and combining it with delivery of ibuprofen could allow for a controlled reduction in inflammation and thus faster recovery and pain relief [7]. To our knowledge, successful doping and release of ibuprofen from pure PLA nanofibrous scaffolds has not previously been reported and multiple challenges were overcome to accomplish this. One of the initial challenges was determining stable electrospinning conditions. This included determining a proper feed rate as well as an appropriate solvent ratio. Using the acid form of ibuprofen ensured a homogenous solution with the PLA dissolved in DMF and CHCl₃. Canto et al. previously reported that the use of the sodium form of ibuprofen resulted in a heterogenous mixture with PLGA and the solvent solution, resulting in beading and spraying during the electrospinning process. The homogenous mixture of ibuprofen and PLA achieved in our approach allowed for a proper viscosity to be maintained, ensuring only minimal beading associated with instability in the electrospinning system, to occur. As SEM images indicated, there was minimal beading that occurred during the electrospinning process (Figure 5.2). It is possible that the fiber diameter increased with respect to an
increasing percentage of ibuprofen (Table 5.1); however, further experiments need to be completed before the result can be validated.

Another challenge presented in this study was the hydrophobic property of PLA. Some investigators have previously attempted to compensate for the hydrophobic properties of PLA by adding additional hydrophilic polymers or utilizing surface treatments. The increase in hydrophilic properties of a surface has shown an increase in endothelial cellular adhesion and growth[12]. PLA has also been paired with PGA to create PLGA for an increased hydrophilicity in order to allow cellular adhesion[9]. Surface treatments have been implemented to increase hydrophilicity of a scaffold as well [13]. The method used in our study was plasma treatment of the surfaces of the ibuprofen loaded PLA scaffold. After plasma treatment, scaffolds were able to sink to the bottom as opposed to non plasma treated scaffolds that would float and water would bead on them. Additionally, HEKs were able to successfully adhere to the ibuprofen loaded PLA scaffold after plasma treatment (Figure 5.6). Further studies on contact angle would need to be completed to fully understand the change in hydrophilic properties [12, 13].

D Velasco et.al.conducted a study in which ibuprofen was loaded into a porous poly(methyl methacrylate)-poly(L-lactic acid) (PMMA-PLA) scaffold created by supercritical CO$_2$[14]. D. Velasco et.al. studied the biocompatibility using fibroblast and found that the addition of ibuprofen supported a higher cellular adhesion. Additionally, Irene Canto et.al.conveyed that HDF successfully adhered to ibuprofen loaded PLGA scaffolds; however, degradation occurred within 6 days[9]. In our study, there was no visible degradation within 2 weeks of the study, which can be attributed to the mechanical integrity
of PLA[7]. There was a significant increase in viable HEKs seeded on ibuprofen-loaded scaffolds at day 14, in comparison to pure PLA at day 14. At 20% ibuprofen, HEK viability was significantly higher than pure PLA at day 14. The decrease in viable HEKs using 30% ibuprofen loaded PLA scaffolds could indicate a cytotoxic effect with the amount of release. 30% ibuprofen loaded PLA scaffolds at 37°C had the highest amount of release (Figure 5.4).

*In vivo* evaluation of ibuprofen loaded scaffolds showed early signs of the degradation in two weeks. It was observed that the scaffolds maintained on wounds for the fully 14 days began to break apart in most cases (representative image in Figure 5.7, middle row). Manual detachment of the scaffolds from the wounds on the last day of the experiment resulted in further disintegration of scaffolds suggesting that their mechanical properties have weakened. Vieira et. al. studied the mechanical properties of PLA during in vitro degradation and reported a direct correlation between the mechanical strength and molecular weight of PLA [15]. Histological analyses of scaffolds kept on the wounds for 14 days indicated that the cells migrated throughout the depth of the scaffolds, as desired (Figure 5.9) potentially through pores that would have enlarged after two weeks of degradation. Growth of cells into the scaffolds further confirms our positive in vitro biocompatibility results. Weir et. al. also reported the biocompatibility of PLLA during the first stage of degradation (less than 4 weeks) [16].

The murine incision wound model used in our *in vivo* analysis heals naturally through the regeneration of new skin and contraction of old skin. However, it is preferred to have accelerated skin regeneration with minimal wound contraction. One of the results of wound contraction is undesired scarring [17]. Interestingly, wounds covered with scaffolds revealed
less contraction compared to untreated control wounds. Specifically the acellular scaffolds significantly reduced wound contraction (Figure 5.8). However, skin regeneration appeared slightly less for the wounds treated with acellular scaffolds relative to untreated controls and cell-seeded scaffolds. Histological analyses revealed a thinner regenerated skin on these wounds. Formation of blood vessels, an indicator of good quality wound healing, was less for acellular scaffolds as well. Cell seeded scaffolds on the other hand supported blood vessel formation while also reducing wound contraction. Immunohistological analysis further confirmed enhanced formation of epidermal layer using cell seeded scaffolds relative to acellular scaffolds. Finally, proliferating cells stained with Ki 67 were greater for scaffold treated wounds relative to untreated controls.

5.8 Conclusions

The data presented here suggests that a homogenous mixture of ibuprofen and PLA can be electrospun to create an ibuprofen loaded PLA nanofibrous scaffold for controlled ibuprofen release. $^1$H NMR confirmed that the chemical integrity of the ibuprofen was maintained after being exposed to a high voltage source. SEM images indicated that the fiber diameter increases with an increasing concentration of ibuprofen, but that the desired nanofibrous morphology is maintained at all concentrations. The release rate of ibuprofen from ibuprofen-loaded scaffolds depends on the initial concentration of this drug and the temperature of release medium. The higher temperature (37°C, body temperature) increased the degradation rate of the scaffold causing a greater amount of ibuprofen release at 37°C. Preliminary in vitro results suggest that the ibuprofen loaded scaffolds were biocompatible.
The optimum concentration of ibuprofen that most supported the viability and metabolic activity of human epidermal keratinocytes for those concentrations tested in this study was found to be 20 wt% to PLA ratio. *In vivo* evaluation of PLA scaffolds containing 20% ibuprofen further confirmed the biocompatibility of such scaffolds; and, it was observed that the cells could migrate throughout the depth and width of the scaffolds over a 14 day experimental period. Covering wounds with acellular ibuprofen loaded scaffolds favorably reduces wound contraction but might delay skin regeneration. However, seeding these scaffolds with human epidermal keratinocytes and human dermal fibroblasts appears to both assist in regeneration of skin and reduce wound contraction.
REFERENCES


Chapter 6

Summary and Conclusions

6.1 Conclusions

This research focused on the development of polymeric nanofibers as drug carriers for wound healing and tissue engineering applications. The goal of this body of work was to regulate and control the release rate of a variety of compounds from nanofibers as desired for multiple clinical applications. In the first chapter, different parameters that can be controlled to change the release profile of drugs from nanofibrous scaffolds were reviewed. These parameters were generally categorized into three areas: fibers, drugs, and release medium with critical aspects of each parameter investigated in depth throughout the body of the dissertation. In particular, fiber morphology was evaluated in the second chapter; drug concentration in chapters three and five; drug properties in chapter four; and release medium temperature in chapter five.

In addition to varying different parameters to control the release rate of drugs from nanofibrous scaffolds, the ability to incorporate different drugs within the nanofibers was also empirically investigated. Different chapters of this dissertation focus on loading a particular drug in polylactic acid electrospun fibers for a specific application. In the second chapter, tricalcium phosphate (TCP) nanoparticles, as an osteoconductive compound, were incorporated in electrospun fibers having different morphologies: regular single component fibers, porous fibers, and core-sheath fibers. It was concluded that the fiber morphology determined the release profile of TCP from the fibers and subsequently influenced
proliferation and osteogenic differentiation of human adipose derived stem cells (hASC) seeded on the TCP-loaded fibrous scaffolds. Human ASC seeded on porous fibers, which exhibited the highest release rate, exhibited the greatest osteogenic differentiation compared to hASC seeded on single component or core-sheath fibers.

The third and fourth chapters focused on delivery of compounds for wound healing in a highly infected wound site. In particular, the ability to incorporate different silver based antimicrobial agents in fibers was extensively evaluated. The goal of this body of work was to deliver silver ions to infected wounds at an effective concentration that would inhibit bacterial growth without causing cytotoxicity to host cells; and to accomplish this without the use of silver nanoparticles. Silvadur ET, a polymeric solution containing silver nitrate, was investigated first and described in Chapter 3. The concentration of silver in the Silvadur ET solution was optimized for its antibacterial/antimicrobial capabilities while maintaining viability of human skin cells. The findings from those studies indicated that specific concentrations of Silvadur ET that were loaded in nanofibrous scaffolds exhibited antimicrobial efficacy while maintaining viability of human epidermal keratinocytes and human dermal fibroblasts in vitro and not inducing significant inflammatory responses in host tissue in vivo, as evaluated in a porcine skin model. Another form of silver was then incorporated and evaluated in the custom PLA nanofibers: highly porous silver microparticles. It was found that these micron size particles could be incorporated in the custom nanofibers. The large size of these particles resulted in their exposure on the surface of the fibers as opposed to embedded within the nanofibers as is typical with silver nanoparticles. As a result of this surface exposure, silver microparticle loaded fibrous
scaffolds exhibited a higher silver ion release rate than silver nanoparticle loaded fibrous scaffolds. The antimicrobial and cytotoxic properties of the silver microparticles were evaluated in a newly designed co-culture system of keratinocytes and *Staphylococcus aureus* bacteria. The preliminary results were promising and confirmed the potential use of these scaffolds for treatment of highly infected wound site.

Chapter 5 continued with wound healing investigations but focused on the incorporation of Ibuprofen as an anti-inflammatory drug for wound healing applications. For the first time, the acidic form of Ibuprofen was dissolved into PLA solution to obtain a uniform dispersion of the drug in solution. PLA fibers with different concentrations of Ibuprofen were electrospun and the effects of drug concentration on release profile and proliferation of human epidermal keratinocytes were evaluated. PLA scaffolds with an Ibuprofen concentration that promoted human epidermal keratinocyte proliferation *in vitro* were tested in an incision wound model in nude mice. It was concluded from this study that the delivery of Ibuprofen at a specific concentration through nanofibrous scaffolds can increase the proliferation of keratinocytes. Further, Ibuprofen-loaded PLA scaffolds that were also seeded with human skin cells further promoted healing of incision wounds by decreasing wound contracture and helping with skin regeneration. Although these studies focused on delivery of a few, specific drugs and compounds through PLA scaffolds, we believe such structures can be doped with variety of other drugs, pharmaceuticals, and/or other compounds that need to be delivered to the target site gradually at a controlled rate.
6.2 Recommendations for future research

In the *in vivo* study described in Chapter 3, it was found that the bacterial concentration was not high enough to stop long term healing of the wounds naturally without any antimicrobial intervention. Therefore, while our antimicrobial scaffolds proved to immediately kill and inhibit bacterial growth in all wounds and kept the wounds free of infection throughout the entire experimental duration, the animals could also heal naturally after an extended period of time. Specifically, although the untreated wounds were immediately infected and remained infected for 5 days, after 7 days, the pigs were able to clear the infection themselves. Therefore, long term efficacy of our scaffolds could not be determined in that pilot study. While the preliminary data generated was very exciting, future studies should investigate a model where the pig cannot clear the infection naturally after an extended duration.

The study described in Chapter 4 investigated the antimicrobial efficacy and cytotoxicity of nanofibers doped with only 0.5% highly porous silver microparticles. Although our results proved the antimicrobial properties of these fibers, complete bacterial growth inhibition was not observed. Increasing the concentration of silver microparticles can help with improving the antimicrobial properties of such scaffolds; however, it can also cause further cytotoxicity to the host skin cells exposed to the scaffolds. Future work should investigate optimization of silver microparticle concentration in fibers while adjusting the initial concentration of bacteria in the co-culture system. Ideally, the concentration of silver microparticles that is non-toxic to mammalian cells but highly toxic to bacterial cells should be determined. Once this is determined, the next step would be to test these scaffolds *in vivo* in burned or infected wound models.
In Chapter 5, the effectiveness of Ibuprofen-loaded scaffolds in wound healing applications was evaluated in a full-thickness incision wound using a nude mice model. According to our results, such wounds can heal naturally in two weeks in nude mice. Considering the slow rate of degradation for PLA (full degradation usually requires at least 6 months depending on molecular weight evaluated), the use of these scaffolds as skin substitutes may be challenging. One of the important characteristics of skin substitutes is that their degradation rate be matched as closely as possible to the rate of skin regeneration so that the scaffolds may mechanically support the wound during the healing process and gradually degrade before the completion of new skin formation. Future studies could be conducted to optimize the degradation rate of scaffolds created in this body of work by copolymerizing PLA with different ratios of a polyethylene oxide, with such ratios to be varied dependent on the desired degradation rate.